GLUCOSE-6-PHOSPHATASE

Its structure, function and regulation
in relation to blood glucose homeostasis

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ABBREVIATIONS USED IN TEXT

AntiT1	Antibody raised to fractions isolated by DIDS/Sepharose affinity chromatography
DIDS	4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(ß-aminoethyl ether) N,N,N'N' -Tetraacetic acid
ER	Endoplasmic reticulum
μg	microgram
FPLC	Fast pressurised liquid chromatography
g	gram
G6P	Glucose-6-phosphate
G6Pase	Glucose-6-phosphatase
GSD	Glycogen storage disease
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
IgG	Immunoglobulin G
kDa	kilo Dalton
l	Litre
M	Molar
M6P	Mannose-6-phosphate
mg	milligram
ml	Millilitre
mM	Millimolar
NTA	Nitrilotriacetic acid
PAGE	Polyacrylamide gel electrophoresis

PP	Pyrophosphate
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SH	0.25M sucrose + 25mM HEPES buffered to pH 7.4
T1	Hepatic microsomal glucose-6-phosphate transport protein
T2	Hepatic microsomal phosphate/pyrophosphate transport protein
T3	Hepatic microsomal glucose transport protein
TBS	Tris buffered saline

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Finally I want to thank my wife and family for their support.

DECLARATION

I declare that this thesis is my own composition. The work presented in Chapter 3 was collected by me as part of a collaborative team - I performed all the endocrine testing and the percutaneous liver biopsises. All the other work was done solely by myself. This thesis has not been submitted in candidature for any other degree, diploma or professional qualification.

JOHN PEARS

August 1992

ABSTRACT OF THESIS

Glucose-6-phosphatase - its structure, function and regulation in relation to blood glucose homeostasis

Hepatic glucose-6-phosphatase (G6Pase) catalyses the final step in blood glucose production by the liver. It is a multicomponent system:the catalytic subunit is on the luminal surface of the endoplasmic reticulum membrane; and there are transport proteins for glucose-6-phosphate, inorganic phosphate and glucose across the ER membrane; and there is a calcium-binding stabilising protein associated to the catalytic subunit. In fasted and diabetic humans (and rat models) kinetic analysis has shown that the capacity of the glucose-6-phosphate transport protein is the rate-limiting step in G6Pase activity, making this protein vital in controlling hepatic glucose output. However, deficiency of any part of this system will lead to hypoglycaemia and other possible metabolic upsets (type 1 glycogen storage diseases). The structure and known regulation of the G6Pase system are reviewed in the introduction to this thesis.

The aims of the work presented here are to investigate the human glucose-6-phosphatase system by studying adult patients newly diagnosed with abnormalities of the G6Pase system, and tissues not previously proven to contain G6Pase in healthy adults thereby improving understanding of the enzyme, its regulation and physiological role and to look for a tissue more accesible than liver in which to study human G6Pase activity.

A unique series of eight adult patients each with an abnormality of hepatic G6Pase (two with previously unrecorded defects) is presented and the features of these cases are discussed with reference to the existing literature on type 1 glycogen storage

diseases. The cases demonstrate how difficult it can be to prove hypoglycaemia in adults; the diversity of presenting symptoms and signs; the use of a screening test (blood glucose response to a 1mg intramuscular dose of glucagon) for such patients; and the benefits of developing reliable assays for the protein components of the G6Pase system. This series of patients also give further clues to the physiological role of glucose-6-phosphatase in extra-hepatic tissues and the regulation of the hepatic G6Pase system.

The diagnosis and subsequent follow-up of the above patients would have been eased by being able to study a more accessible tissue than liver. Intestinal mucosa and neutrophils have been described as abnormal in G6Pase deficiencies. Therefore G6Pase activity was sought in these tissues from normal adult humans.

Confirmation of the presence of the G6Pase catalytic subunit in intestinal mucosa is presented; and data from neutrophils which is highly suggestive of catalytic subunit being present, but little demonstrable activity due to proteolysis. The use of either of these tissues to study human glucose-6-phosphatase activity is therefore still not reliable. Possible physiological roles for glucose-6-phosphatase in both intestinal mucosa and neutrophils is discussed.

I also set out to study the structure and regulation of the glucose-6-phosphate transport protein with the (long-term) view of understanding and down-regulating the increased hepatic glucose output seen in diabetes mellitus.

Studies of the G6P transport protein required the development of new techniques (such as microsomal transport assays, sodium-free systems and affinity chromatography).

Little has been known of the glucose-6-phosphate transport protein (T1), despite its obvious importance in the pathogenesis and possible site of pharmacological manipulation in diabetes mellitus. I have demonstrated, for the first time, that glucose-6-phosphate co-transports with sodium across the endoplasmic reticulum membrane and that the normal transport of both requires a divalent metal ion

(probably calcium). Following from this is the first evidence of *in vitro* pharmacological manipulation of T1 with the sodium channel inhibitor amiloride. Early work to isolate and purify T1 using affinity chromatography techniques is also presented, and data using antibody raised to a protein isolated in this fashion is shown. The protein isolated is compared to an earlier protein which was thought originally to be T1.

Finally I look forward to the future and the continuation of this research.

CHAPTER 1

INTRODUCTION

The liver plays a vital role in controlling glucose homeostasis in mammals (Nordlie 1985). In the post prandial state the liver takes up glucose for storage as glycogen or metabolism. Under fasting conditions the liver produces glucose to maintain blood glucose levels. In times of acute stress the liver rapidly releases glucose to maintain blood glucose levels for use as a fuel by tissues which cannot produce glucose - eg brain and muscle (Nordlie 1985). In the liver the final step in glucose production via both the glycogenolytic and gluconeogenic pathways is the hydrolysis of glucose-6-phosphate by a substrate specific phosphatase (Figure 1.1). This phosphatase is glucose-6-phosphatase (EC 3.1.3.9). Any compound, deficiency or disease state affecting glucose-6-phosphatase structure or function will, therefore, affect glucose homeostasis by influencing the final step in both glycogenolysis and gluconeogenesis.

The work in this thesis is concerned with this final step in glucose production within the liver - the hydrolysis of glucose-6-phosphate by the enzyme glucose-6-phosphatase.

In this opening Chapter I will summarise the knowledge and the theories concerning glucose-6-phosphatase as they were when my work started.

Despite its crucial position in glucose homeostasis and many years of study, the biochemistry of glucose-6-phosphatase is not well understood. This is mainly due to the fact that the enzyme is intimately associated with intracellular membranes, and is very pH and temperature sensitive.

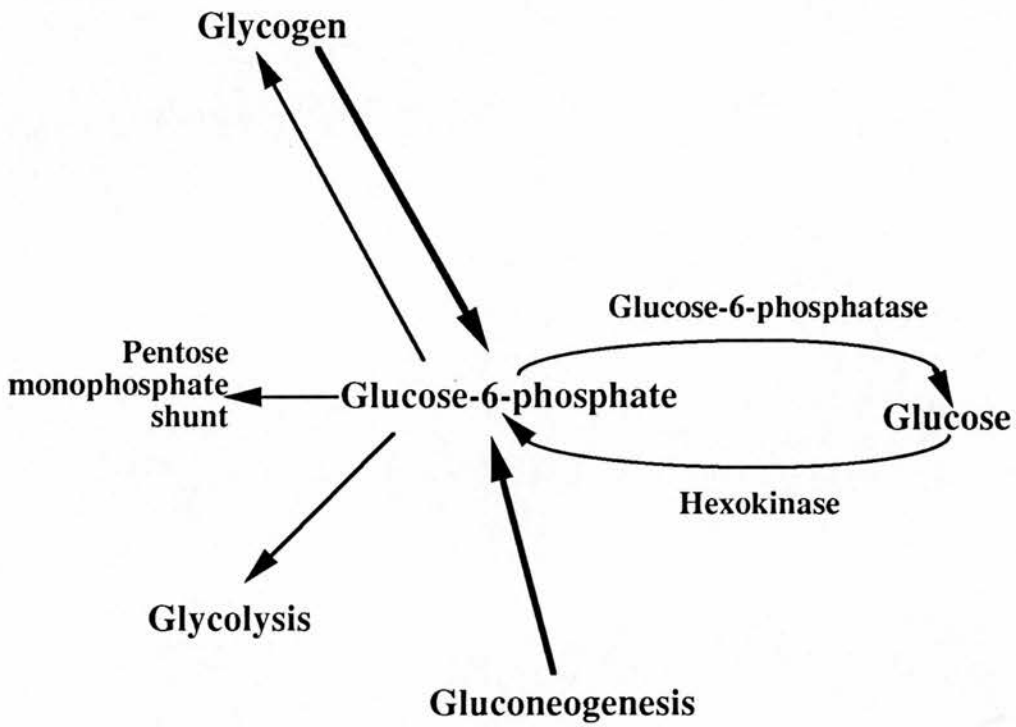


Figure 1.1

A representation of the pathways of metabolism in hepatocytes showing the central position of glucose-6-phosphate in a number of pathways, and the terminal step in glucose production by glycogenolysis and gluconeogenesis being catalysed by glucose-6-phosphatase. Also indicated is the substrate cycle between glucose-6-phosphate and glucose which occurs continuously and which is a site of control of hepatic glucose output and uptake.

1.1 HISTORICAL BACKGROUND

1.1.1 Multifunctional potential of glucose-6-phosphatase

In vivo the glucose-6-phosphatase enzyme is very substrate specific. *In vitro* however glucose-6-phosphatase can catalyse many reactions. Some of these reactions can be employed to study glucose-6-phosphatase activity in tissue samples.

Glucose-6-phosphate + H_2O	------→ Glucose + P_i	(1)
Mannose-6-phosphate* + H_2O	------→ Mannose + P_i	(2)
Pyrophosphate (PP_i) + H_2O	------→ $2P_i$	(3)
Glucose + PP_i	------→ Glucose-6-phosphate + P_i	(4)
Glucose + Carbamyl-phosphate	----→ Glucose-6-P + NH_3 + CO_2	(5)

* Many other phosphate salts (notably glucosamine-6-phosphate and carbamyl-phosphate but not glucose-1-phosphate) can also be hydrolysed by glucose-6-phosphatase.

The specificity of glucose-6-phosphatase for different phosphate salts was recognised relatively early after the discovery of the enzyme (Beaufay & De Duve 1954a). Not until much later was this specificity satisfactorily interpreted along with kinetic analyses to produce the models of glucose-6-phosphatase described below. The physiological role of glucose-6-phosphatase has been subject to much speculation. There is no doubt that reaction (1) above occurs *in vivo*. Reactions (2) and, probably, (3) do not. The physiological role of the microsomal pyrophosphate transport protein (T2) is unclear; it is possible that pyrophosphate is required for another intraluminal process. It has been proposed that reactions (4) and (5) can occur in hepatocytes (Nordlie 1985) in conditions of glucose excess. The Km of glucose-6-phosphatase for glucose in the latter two reactions *in vitro* is 80-90mM (Arion & Nordlie 1964) and *in vitro* 120mM glucose has been used to assess the

phosphotransferase activity of glucose-6-phosphatase (Nordlie 1976). It seems unlikely that this concentration of glucose will occur *in vivo*. However it can be argued that as microsomes are artefacts, the conditions needed for reactions (4) & (5) to proceed *in vitro* do not reflect those necessary *in vivo*.

Glucose-6-phosphatase *in vivo* also shows multifunctionality. The most convincing evidence for this was the demonstration that microsomes prepared from the liver of a patient with type 1a glycogen storage disease (complete absence of glucose-6-phosphatase activity) could not perform any of the reactions attempted: glucose-6-phosphate and sodium pyrophosphate hydrolysis; and pyrophosphate-glucose and carbamyl phosphate-glucose phosphotransferase (Hefferan & Howell 1977).

The multifunctionality of the glucose-6-phosphatase enzyme has been exploited in assaying microsomes prepared from patients (especially reactions 1-3 above) and is important in understanding the biochemistry of glucose-6-phosphatase.

1.1.2. Biochemistry

Cori and Cori showed in 1938 that glucose was produced in the liver by the action of a phosphatase (Cori & Cori 1938) and in 1945 Fantl and Rome demonstrated that the final step in hepatic glucose production involved the specific hydrolysis of glucose-6-phosphate. From this time on a lot of work was done trying to isolate and characterise the glucose-6-phosphatase enzyme (for a detailed early review of this work see Ashmore & Weber 1959). Much of this pioneering work has been confirmed and used to formulate the present day ideas about glucose-6-phosphatase. For instance the pH optimum of the enzyme was shown to be between 6 and 7 (eg Ashmore *et al* 1954) and has subsequently been shown to be 6.5; the marked sensitivity of the enzyme to temperature changes (Cori & Cori 1952 and Beaufay *et al* 1954); and (very importantly) the inactivation of the glucose-6-phosphatase enzyme by incubation at pH 5 (Beaufay & de Duve 1954a) became clear.

Unfortunately some of this data (especially the last work mentioned above) has

subsequently been ignored by many workers. This has lead to numerous erroneous claims in the literature of "glucose-6-phosphatase" activity where data have been confused by the presence of non-specific phosphatase activity, which could easily have been corrected for using careful experimental technique.

The intracellular distribution of glucose-6-phosphatase in the hepatocyte was first recognised in 1951: using the then new technique of differential centrifugation Hers *et al* (1951) demonstrated that glucose-6-phosphatase activity was in the microsomal fraction. Another important piece of early work concerned the effects of detergents on microsomal glucose-6-phosphatase activity (Beaufay & de Duve 1954b). This data showed that at low concentrations of detergent there was activation of the enzyme's activity but inhibition at higher concentrations. This was the first demonstration of the "latency" of microsomal glucose-6-phosphatase activity. It is the mechanism of this "latency" which has stimulated much of the investigation into and caused much of the controversy surrounding microsomal glucose-6-phosphatase.

1.1.3 The latency of microsomal glucose-6-phosphatase.

The latency (or "detergent sensitivity") of microsomal glucose-6-phosphatase is the percentage of specific glucose-6-phosphatase activity which is not expressed in an assay of untreated microsomal preparations, but which is expressed when the integrity of the microsomal membrane is disrupted. Latency is caused by the catalytic site of the enzyme being buried within the lipid component of the endoplasmic reticulum membrane.

Latency is calculated from the following equation:

$$\text{Percentage Latency} = 100 \times \frac{[\text{d m}] - [\text{i m}]}{[\text{d m}]}$$

Where dm and im are the activity of glucose-6-phosphatase in disrupted and intact microsomes respectively. "Disrupted" microsomes are those treated, for example, with detergent, by sonication or with histone 2A and "intact" microsomes have had their activity "corrected" for intactness (see below).

The latency of microsomal glucose-6-phosphatase is variable, and depends upon a number of factors: the method of microsomal preparation; how old the preparation is (the apparent latency falls as microsomes become older due to a decrease in the glucose-6-phosphatase activity of disrupted microsomes because of temperature inactivation of the enzyme); and how often the preparation has been frozen and thawed (this reduces the latency of untreated microsomes by disrupting the microsomal membrane). The latency of hepatic microsomal glucose-6-phosphatase activity is also altered by the metabolic state of the animal from which the microsomes are prepared: latency is increased in microsomes prepared from fasting animals compared to fed ones (Nordlie *et al* 1968) and is increased again in diabetic animals (Nordlie *et al* 1979b); in glucocorticoid-treated animals the latency is decreased (Arion *et al* 1976b).

Microsomes are an artefact and comprise vesicular fragments of endoplasmic reticulum surrounded by a single continuous membrane (Palade & Siekevitz 1956). During the preparation of microsomes there is no guarantee that the endoplasmic reticulum membrane vesicles will be intact. This would result in an apparent decrease in latency of microsomal glucose-6-phosphatase activity as the apparent activity of untreated microsomes will be higher. Knowing the percentage latency of untreated microsomal preparations is important in correctly interpreting kinetic data of hepatic microsomal glucose-6-phosphatase activity. This percentage of "correctly" formed microsomes can be assessed by exploiting the substrate specificity of glucose-6-phosphatase in untreated microsomes. It was recognised in 1955 that, for example, the hydrolysing ability of glucose-6-phosphatase for

mannose-6-phosphate and glucosamine-6-phosphate was lower than for glucose-6-phosphate. It is likely that these data were obtained using microsomes not fully "intact" as the mannose-6-phosphate hydrolysing activity he demonstrated was much higher than was subsequently found in microsomes known to be "intact" (Arion *et al* 1972). Using detergent treated microsomes, however, the hydrolytic activity of glucose-6-phosphatase is identical for both glucose-6-phosphate and mannose-6-phosphate substrates (Arion *et al* 1972). The hydrolysing activity of untreated microsomes with mannose-6-phosphate substrate compared to this activity measured in disrupted microsomes has been adopted as a reliable way of assessing the intactness or otherwise of a microsomal preparation which should then be allowed for in kinetic studies (Arion *et al* 1976a). As the Km of glucose-6-phosphatase in intact microsomes is much higher than that in disrupted microsomes, therefore only a small proportion of disrupted microsomes will markedly alter the apparent kinetics of the hydrolysis of glucose-6-phosphate.

The calculation of "intactness" of a microsomal preparation (expressed as a percentage of the total microsomal population) is calculated from the following equation:

$$V_{im} = V_{um} - V_{dm} \times \frac{\text{Mannose-6-phosphatase}_{um}}{\text{Mannose-6-phosphatase}_{dm}}$$

Where V_{im} is the velocity of the reaction in microsomes with intact membranes, V_{um} is the velocity of the reaction in untreated microsomes and V_{dm} the velocity in fully disrupted microsomes.

Data using permeablised hepatocytes has shown differences in kinetic constants of glucose-6-phosphatase to microsomes (Jorgenson & Nordlie 1980 and McEwen & Arion 1985): the main difference is that the latency of the enzyme and the Vmax do not increase as much as they do in microsomal preparations moving from fed to

starved to diabetic animals. However there are some concerns about this data, mainly that the methods used to permeabilise microsomes may have damaged the ER too.

The explanation of the phenomena of latency and substrate specificity have occupied many workers on glucose-6-phosphatase for many years and has given rise to one of the most controversial aspects of the enzyme: its structure in, and relationship to, the endoplasmic reticulum membrane.

1.1.4 Two theories on the structure of glucose-6-phosphatase.

Two theories have arisen - largely based upon the interpretations made by a number of individuals on data available at the time - to explain the latency and substrate specificity of microsomal glucose-6-phosphatase. These are the "Conformational" and "Multicomponent Transporter System" models, and will be discussed individually. Initially these two theories were seen by many as mutually exclusive, but it may be that both are, to differing extents, correct.

The presence of latency of glucose-6-phosphatase activity which can be removed by detergent treatment and the specificity of the hydrolysing capability of the enzyme in untreated microsomes for glucose-6-phosphate suggests that the catalytic activity is contained within a phospholipid membrane and that the presence of the membrane in some way constrains the catalytic activity.

Once the microsomal model of glucose-6-phosphatase has been clarified (a difficult enough task) the *in vivo* model of glucose-6-phosphatase will then have to be defined.

a) The Conformational Model of glucose-6-phosphatase

The basic concept of this model is that glucose-6-phosphatase catalytic activity is buried in the endoplasmic reticulum membrane and that changes in the local

membrane microenvironment result in the interactions of the catalytic site with its various substrates. Initially this model suggested that the catalytic site of the glucose-6-phosphatase was on the outside of the endoplasmic reticulum *in vivo* (but became buried within the membrane during the preparation of microsomes). The binding of the substrate caused a conformational change whereby the enzyme-substrate complex crossed the ER membrane and glucose was then produced inside the lumen of the endoplasmic reticulum (Stetten & Burnett 1967) which was in continuity with the extracellular space where the glucose was required. Therefore there is no transport of glucose-6-phosphate or reaction products across the ER membrane.

It had earlier been proposed that this conformation would favour the synthesis of glucose-6-phosphate from extracellular glucose (Siekevitz 1958) - one of the postulated roles of glucose-6-phosphatase (see above). However as pointed out above, the synthesis of glucose-6-phosphate by glucose-6-phosphatase will only occur (*in vitro*) at very high glucose concentrations which are very unlikely to occur *in vivo*. Stetten and Burnett suggested that treating microsomes with detergent restored the *in vivo* conformation of the enzyme in the endoplasmic reticulum membrane. However this is difficult to believe as the activity of detergent treated microsomal glucose-6-phosphatase is very high and (by this early model) relatively uncontrolled and would presumably result in a huge out-pouring of glucose by the liver. It is likely that glucose-6-phosphatase activity is under extra cellular control as it catalyses one side of a substrate cycle (see Figure 1.1 and discussion below).

Subsequently the conformational model has been modified from that described by Stetten and Burnett to that summarised by Schulze *et al* in 1986. The glucose-6-phosphatase molecule now is interpreted as a water-filled tube through the hydrophobic endoplasmic reticulum membrane. Much of the basis of this theory is inferred from the interpretation of studies of inhibitors of glucose-6-phosphatase

activity (sulfhydryl reagents and organic mercurials). The purification of this single protein has not been achieved.

This model disregards most of the kinetic observations of glucose-6-phosphatase activity and makes understanding the type 1 glycogen storage disease variants very difficult.

b) The Multicomponent Transport Theory of Glucose-6-phosphatase

The basis of this model is that the catalytic subunit of glucose-6-phosphatase lies on the luminal surface of the endoplasmic reticulum and that there are individual transport proteins to permit the passage of substrate and reaction products across the endoplasmic membrane. This model of glucose-6-phosphatase was first suggested after careful kinetic studies on microsomal glucose-6-phosphatase and the substrate specificity conferred by the intact microsomal membrane (Arion *et al* 1972). Arion's data showed clearly that in intact microsomes the hydrolysis of glucose-6-phosphate was more rapid than the hydrolysis of mannose-6-phosphate. Further work in Arion's laboratory using sulfhydryl poisons lead to the evolution of the multicomponent model as it has become understood (Wallin & Arion 1973, Arion *et al* 1975 and Arion *et al* 1980b).

There are now three postulated transport proteins across the ER membrane for glucose-6-phosphate, phosphate and glucose (named T1, T2 and T3 respectively). In 1982 a 21kDA protein was isolated from microsomes, antibodies to which immunoprecipitated >90% glucose-6-phosphatase activity from microsomes (Burchell & Burchell 1982) but which was subsequently shown to be a regulatory protein closely associated with the glucose-6-phosphatase catalytic subunit (Burchell *et al* 1985). Ann Burchell has called this protein "stabilising protein" as (*in vitro*) it stabilised glucose-6-phosphatase activity (Burchell *et al* 1985).

Figure 1.2 shows the structure of the glucose-6-phosphatase multicomponent model as it was visualised when my work started. The catalytic subunit is associated to the luminal surface of the endoplasmic membrane and the 21kDa stabilising protein.

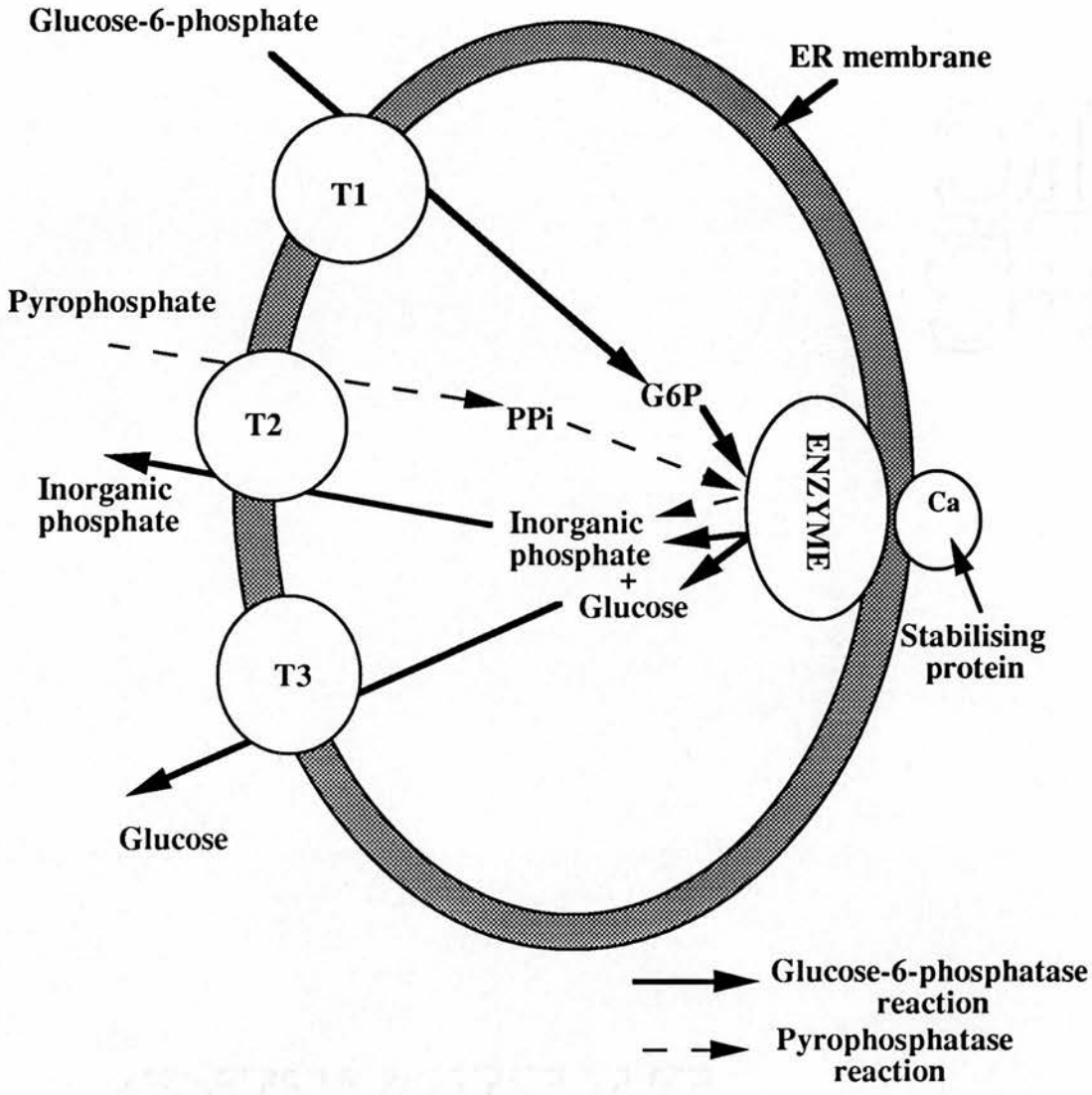


Figure 1.2

Diagrammatic representation of the multicomponent transporter model of hepatic microsomal glucose-6-phosphatase as it was visualised when this thesis started. The catalytic subunit (enzyme) is associated to the luminal surface of the ER membrane and a calcium-binding stabilising protein. There are three transport proteins to allow the influx of substrate glucose-6-phosphate and the efflux of reaction products phosphate (which counter-transports with pyrophosphate) and glucose from the lumen of the ER; named T1, T2 and T3 respectively.

The model suggests three transport proteins to allow substrate (glucose-6-phosphate) to cross into the lumen of the ER, and the reaction products inorganic phosphate (P_i) and glucose to pass back into the cytoplasm. These transport proteins have been called T1, T2 and T3 respectively. The inorganic phosphate transport protein (T2) was also postulated to counter-transport pyrophosphate.
The ultimate proof of existence of the multicomponent model of glucose-6-phosphatase will be the isolation of the proteins involved.

In the rest of this thesis the Multicomponent Transport Model of glucose-6-phosphatase activity is assumed

1.1.5 Purification of the proteins of the glucose-6-phosphatase system

Purification of the active microsomal glucose-6-phosphatase protein has proved difficult. The protein has proved very difficult to dissociate from the microsomal membrane and all the detergents used to achieve the dissociation also inhibit glucose-6-phosphatase activity. Once dissociated from the ER membrane the glucose-6-phosphatase activity is very unstable in a time and temperature dependent manner. This instability may reflect dissociation of the enzyme activity from the stabilising protein as much as from the membrane environment.
Several claims of the purification of the glucose-6-phosphatase protein have been made from a variety of tissues and involve a variety of differing sizes of peptide: Anchors and Karnovsky isolated a 28kDa protein from rat brain which reportedly retained glucose-6-phosphatase activity and similar kinetic characteristics to the microsomal enzyme (Anchors & Karnovsky 1975); Reczek and Villee claimed that a 58-64kDa protein they isolated from human liver and placenta was the glucose-6-phosphatase enzyme, but it only displayed a small increase in glucose-6-phosphatase activity (Reczek & Villee 1982). No further data has been forthcoming about these proteins: such as structures, sequences or studies with specific antibodies. Therefore

it seems unlikely that they do in fact represent the glucose-6-phosphatase catalytic protein. Furthermore, subsequent work has shown clearly that specific glucose-6-phosphatase activity is not present in placenta, but that high levels of non-specific phosphatases are (Chen & Kato 1985 and Barash *et al* 1991).

Ann Burchell has tried over a number of years to purify a single glucose-6-phosphatase catalytic protein without success. The best attempt resulted in the isolation of 5 peptides identified by SDS-PAGE. Studies on these five peptides (which together retained glucose-6-phosphatase activity) revealed the presence of the 21kDa stabilising protein (Burchell *et al* 1985) and studies on microsomes identified the catalytic subunit as part of a 36.5kDa doublet (Countaway *et al* 1988). Polyclonal antibodies raised to this doublet used to immunostain liver microsomes from a patient with type 1a glycogen storage disease (complete absence of glucose-6-phosphatase activity) showed this 36.5kDa protein to be absent, thus confirming that this doublet did indeed represent the glucose-6-phosphatase catalytic protein (Burchell *et al* 1988b).

Progress on the purification of the transport proteins has been slow. In 1982 Zoccoli *et al* published work regarding a 54kDa protein that they had identified and which they considered to be the microsomal glucose-6-phosphate transport protein T1 (Zoccoli *et al* 1982). In 1986 (Countaway & Arion) this protein was shown not to be T1 and the original group reported nothing more on this protein. No other reports regarding the structure of the T1 transporter have been published. The T1 transport protein is the subject of Chapter 5 where it is discussed in greater detail. In contrast the pyrophosphate/phosphate antiporter T2 has been shown to be a 37kDa peptide (Waddell *et al* 1988). No data was forthcoming regarding the microsomal glucose transport protein T3 when my work started.

Studies of the genetic deficiencies of glucose-6-phosphatase have resulted in major advances in the understanding of the structure of the enzyme and its associated proteins.

1.1.6 Genetic Deficiencies of Hepatic Glucose-6-phosphatase

The first description of a human condition involving an apparent abnormality of hepatic glucose production was made in 1929 by von Gierke when he described the autopsy findings on two children with clinical histories compatible with a glycogen storage disease. In 1952 Cori and Cori demonstrated for the first time the absence of glucose-6-phosphatase activity in the liver of a patient with hypoglycaemia and hepatomegaly (Cori & Cori 1952). The Cori's called this disease "von Gierke's" disease in deference to the 1929 report. However it seems likely in retrospect that the cases described in 1929 had a deficiency of the debranching enzyme rather than of glucose-6-phosphatase. The use of eponymous names for the glycogen storage diseases is confusing, and throughout this thesis the numerical classification as described in Hers *et al* 1989 will be used. Thus the deficiencies of glucose-6-phosphatase are the type 1 glycogen storage diseases (GSDs).

Several clinical pictures have been recognised in patients with type 1 glycogen storage disease and these will be discussed in more detail in Chapter 3. Briefly there are patients who have severe hypoglycaemia, huge livers and kidneys (which are full of glycogen), hyperuricaemia, hyperlipidaemia, a bleeding tendency and high plasma lactate levels (especially on fasting) and who tend to die young of hypoglycaemic coma, renal failure or hepatocellular carcinoma. There is no demonstrable glucose-6-phosphatase activity in either intact or disrupted microsomes made from the livers of these patients. This pattern is called type 1a glycogen storage disease. This was thought to be a severe condition which was diagnosed in early life, but more recently partial forms of the disease have been demonstrated in adults (Burchell *et al* 1987).

Other patients have all the above signs and a tendency to bacterial sepsis. These subjects often have demonstrable abnormalities of neutrophil function (eg Anderson 1981). Study of hepatic microsomal glucose-6-phosphatase activity in these subjects was initially confusing as low levels of activity are found in intact microsomes but

in disrupted microsomes the glucose-6-phosphatase activity is normal or high. However careful kinetic studies by Lange *et al* explained these findings as representing deficiency of the microsomal glucose-6-phosphate transport protein T1 - *ie* type 1b glycogen storage disease (Lange *et al* 1980). Types 1a and 1b glycogen storage disease are diagnosed by assaying intact and disrupted microsomes with both glucose-6-phosphate and mannose-6-phosphate as substrates. Type 1a glycogen storage disease may be confirmed in some cases (but not all) by immunoblot analysis of microsomes using the polyclonal antibody to the catalytic protein. If the microsomal hydrolytic activity for glucose-6-phosphate is present but the patient has excess glycogen stores and recurrent hypoglycaemia then the microsomes (intact and disrupted) must be assayed for hydrolysing activity of pyrophosphate - absent activity in intact microsomes which is present in disrupted microsomes is indicative of type 1c glycogen storage disease: absence of a functional phosphate/pyrophosphate transport protein, T2. This can also be confirmed by immunoblot analysis using the antibody raised against T2 and described by Waddell *et al* (1988). However some type 1a and 1c glycogen storage disease patients do have the relevant immunoreactive protein in hepatic microsomal preparations. There have been (at the time of writing) no descriptions of type 1d glycogen storage disease (absence/deficiency of the microsomal glucose transporter T3). This may be because the existence of T3 is still unproven and/or because there is no clear assay for the capacity of such a protein.

A review of the kinetic methods for diagnosing type 1 glycogen storage disease variants is given in Burchell *et al* 1988a.

The study of these genetic deficiencies of glucose-6-phosphatase is some of the strongest proof for the multicomponent model of glucose-6-phosphatase as described above. It is difficult to conceive how the conformational model of glucose-6-phosphatase can accommodate these data. Unfortunately the diagnosis of all these

abnormalities has to be made on liver tissue as there is no more accessible tissue with definite glucose-6-phosphatase activity to use.

1.1.7 Other observations on glucose-6-phosphatase

In other conditions of abnormal glucose output (such as diabetes mellitus and high circulating corticosteroid levels) glucose-6-phosphatase activity is abnormal. In untreated diabetes mellitus there is increased enzyme activity in disrupted microsomes and increased latency for glucose-6-phosphate and mannose-6-phosphate in intact microsomes (Arion *et al* 1976a and 1980b). Associated with this increase in catalytic activity there is an increase in the amount of catalytic subunit protein (Burchell & Cain 1985). Treatment with insulin in streptozotocin induced diabetes decreases glucose-6-phosphatase activity (Nordlie & Arion 1965, Nordlie 1971 and Burchell and Leakey 1988).

In conditions of glucocorticoid excess, there is increased catalytic activity due largely to a decrease in measured latency for glucose-6-phosphate (Arion *et al* 1976b).

As Figure 1.1 shows, glucose-6-phosphatase catalyses one side of a substrate cycle involving the interconversion of glucose and glucose-6-phosphate. The opposite reaction is catalysed by glucokinase, the activity of which is inhibited by glucose-6-phosphate, but is markedly increased by glucose concentrations in the physiological range 5-10mM. Both glucokinase and glucose-6-phosphatase have been suggested as being continuously but variably active in hepatocytes (Hue 1982). The regulation of glucokinase has been extensively investigated and is complex, as would be expected from the theory that the substrate cycles can respond rapidly to a number of different stimuli (van Schaftingen & Vandercammen 1989, Davies *et al* 1990, Detheux *et al* 1991 and Vandercammen & van Schaftingen 1991). The balance between the activity of the two enzymes therefore determines if there is net uptake by or release of glucose from the liver. As the cycle continuously operates it means

that the response to extra-hepatocyte conditions (such as hormonal stimulation) is very rapid.

1.1.8 Tissue Distribution of Glucose-6-phosphatase

The type 1 glycogen storage diseases are associated with enlargement of the liver and kidney due to increased glycogen stores. Glucose-6-phosphatase activity can be measured in the kidney and the presence of the catalytic subunit has been shown by immunoblotting with the antibody to the 36.5kDa protein described above (Waddell & Burchell 1988). Many other tissues have been reported as containing glucose-6-phosphatase (see Nordlie 1971, Colilla *et al* 1975 and Nordlie & Sukalski 1985 for review) however some of the reviewed data is suspect as the assay techniques employed have often identified non-specific phosphatase activity at such high levels that any specific glucose-6-phosphatase activity is masked. To establish specific glucose-6-phosphatase activity in a tissue it is necessary to demonstrate glucose-6-phosphate hydrolysis in the microsomal fraction of that tissue with the same kinetic and specificity profile as in liver microsomes; incubation of the microsomes at pH 5 and 37°C for 10 minutes should destroy the activity; and there should be a reaction on immunoblot analysis with the antibody to the 36.5kDa catalytic subunit. Using these strict criteria glucose-6-phosphatase activity has been confirmed in the liver and kidney and demonstrated in pancreatic islet cells (Waddell & Burchell 1988) and gall bladder mucosa (Hill *et al* 1989).

The physiological role for the enzyme in all these tissues is still to be clarified and is discussed in more detail in Chapters 3 and 4 below.

The aims of the work described in this thesis were to further investigate abnormalities of glucose-6-phosphatase activity in adult humans; to try and extend knowledge regarding the tissue distribution of the enzyme, at the same time looking

for a more accessible tissue on which to study glucose-6-phosphatase activity *in vivo*; and to study the microsomal glucose-6-phosphate transport protein.

The study of human glucose-6-phosphatase abnormalities is described in Chapter 3 where as well as two new abnormalities, six unusual cases are presented. The importance of correctly assaying microsomal preparations with up to three substrates is clearly demonstrated. In an attempt to further understand the physiology of glucose-6-phosphatase and to find a more accessible tissue than liver in which to study the enzyme *in vitro* I wanted to look at the activity of the enzyme in intestinal mucosa and neutrophils. This work is described in Chapter 4.

The key rate-limiting step to glucose-6-phosphatase activity is the capacity of the glucose-6-phosphate transport protein, T1. Nothing was known of this protein at the start of this thesis, but its presence was inferred from kinetic analysis of glucose-6-phosphatase activity and by its absence in type 1b glycogen storage disease. I wanted to isolate the protein, study its biochemistry and attempt to pharmacologically manipulate its capacity for glucose-6-phosphate transport with the longer-term view of altering hepatic glucose output. This work is described in Chapter 5.

CHAPTER TWO

MATERIALS AND METHODS

2.1 PATIENTS

The cases described in Chapter Three were referred to Ninewells Hospital, Dundee and were investigated under the direct supervision of Dr.Roland Jung, Consultant Physician.

The studies of glucose-6-phosphatase activity in human intestinal mucosa described in Chapter Three were performed on individuals undergoing routine gastrointestinal endoscopy by the endoscopy service at Ninewells Hospital, Dundee.

The studies of glucose-6-phosphatase activity in human blood were performed on venous blood samples drawn from normal volunteers.

Permission for both of these studies had been given by the Tayside Health Board Committee on Medical Ethics.

2.1.1 Biopsies

a) Liver biopsies. These biopsies were performed under local anaesthesia (unless otherwise specified) on conscious patients through the eighth right intercostal space in the mid-axillary line. Biopsies were taken with 'Surecut' (modified Menghini) needles (TSK Laboratories, Japan). Before biopsy all patients gave informed, signed consent. Tests of *in vitro* blood clotting (prothrombin time and kaolin cephalin clotting time unless otherwise specified) and a full blood count (for haemoglobin concentration and platelet count) were routinely carried out before the biopsy. Serum was also cross-matched for use in the case of emergency haemorrhage.

After the biopsy patients rested flat in bed for at least four hours, and were not allowed out of bed for at least twelve hours. During this period of rest regular measurements of pulse and blood pressure were made and assessment of need for analgesia was carried out. A number of the patients experienced mild right upper abdominal/right shoulder-tip discomfort requiring simple oral analgesia (paracetamol). One particularly anxious patient required a single dose of parenteral analgesia (pethidine). No episodes of haemorrhage, peritonism or pleurisy were observed. All patients were fit for discharge from hospital (with instruction to avoid strenuous exercise and heavy lifting for seven days) twenty-four hours after the biopsy.

Samples of liver for histology were transported in phosphate-buffered formaldehyde/phenol-formaldehyde solution (Hopwood *et al* 1990). Paraffin sections were stained with haematoxylin and eosin and PAS. Samples for electron microscopy were fixed in 0.2M cacodylate buffered 3% glutaraldehyde and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 100 CX electron microscope at 60 kV.

Liver pathology for all patients described was examined by Dr. David Hopwood, Consultant Pathologist, Ninewells Hospital, Dundee.

Samples of liver for biochemical analysis were placed immediately in ice-cold 0.25 M sucrose/5 mM HEPES buffered to pH 7.4 (SH pH 7.4). All assays for glucose-6-phosphatase, mannose-6-phosphatase and pyrophosphatase activity were performed on fresh unfrozen tissue within 1 hour of biopsying (see 2.6 below for methods). Estimations of protein and glycogen content were performed on samples which had been frozen at -70°C for a maximum of 48 hours (for assay methods see 2.6 below and Chapter 3 for further details of the patients studied).

b) Gastrointestinal mucosa biopsies. Patients attending the Department of Clinical Measurement, Ninewells Hospital, Dundee for routine out-patient endoscopic examination of the gastrointestinal tract were approached and asked to give

informed consent to biopsying of normal-looking areas of gut mucosa. Each patient was biopsied from only one area of the gastrointestinal tract. No patient had biopsies taken on more than one occasion. All patients were observed by the staff of The Department of Clinical Measurement, Ninewells Hospital after the sample for signs of significant haemorrhage. None were observed.

Samples of mucosa were taken from macroscopically normal areas of gut using standard pinch biopsy forceps through an Olympus endoscope by experienced endoscopists. Five or six biopsies were taken from a single area of gut in each patient giving a total wet weight of 30-50 mg mucosa. The biopsies were collected immediately into ice cold 0.25 M sucrose/5 mM HEPES pH 7.4 containing a cocktail of protease inhibitors (see Table 2.1) or wrapped in aluminium foil and frozen immediately in liquid nitrogen (see 2.5.1). The biopsies were assayed immediately - the processing was started within 15 minutes of the biopsy being taken. See Chapter 4.1 for details of results.

Table 2.1

Protease inhibitor cocktail used to collect samples of intestinal mucosa used for activity measurements.

Protease inhibitor	Final concentration in SH pH 7.4
Trypsin/chymotrypsin inhibitor	0.01 mg/ml
1,10 - phenanthroline	10 mM
Pepstatin A	0.1 mM
Phenylmethylsulphonyl fluoride (PMSF)	0.02 % (w/v)
PMSF in 10% ethanol	

c) Venous blood. Ten ml of peripheral venous blood was drawn (without stasis whenever possible) from healthy volunteers into either tubes containing lithium heparin or into tubes containing 1ml trisodium citrate. The blood cell types were

then separated out immediately as described below (see Chapter 4.2 for details of results). All blood samples were processed within 30 minutes of collection.

2.1.2 Endocrine Testing

Prior to all of these tests an indwelling intravenous cannula was placed into an antecubital fossa vein. The cannula was kept patent with 1ml flushes of saline containing heparin 10 U/ml. The first 1 ml of each blood sample was discarded.

a) Glucagon test One mg of glucagon (Novo Laboratories, UK) was given by intramuscular injection (the deltoid in the non-dominant upper limb was used) at time '0'. The subjects had been fasted overnight. Blood samples were taken at set times (predose, 30, 60, 90 and 120 minutes post injection) for measurement of blood glucose levels. Subjects were then fed and, if out-patients, allowed home. Blood glucose levels were assayed in duplicate using a Beckman Glucose Analyser. Serum insulin levels were measured by radio-immuno assay (kits from Amersham Ltd and Serono were used).

Interpretation A rise in blood glucose of $\geq$4mmol/l was taken as normal (see Discussion in Chapter 3).

b) Oral glucose tolerance test After an overnight fast 75g of glucose dissolved in 200ml water was given as a drink (all drinks were completed within 3 minutes). Samples of blood were taken at set times for glucose and (in case 8) insulin levels (predose, 30, 60, 90, 120, [150 & 180] minutes after the drink - the 150 & 180 minute samples were not routine). The first 1ml of each sample was discarded. After the last sample was drawn the subject was fed and, where appropriate, allowed home.

Interpretation Standard World Health Organisation criteria (Report of the WHO Study Group on Diabetes Mellitus 1985) were used to interpret the blood sugar changes of diabetes/impaired glucose tolerance. The interpretation of the blood glucose levels in terms of hypoglycaemia were made for individual cases. The

serum insulin levels (when measured) were interpreted, likewise, in the context of each case.

c) Intravenous glucose tolerance test This was performed only on case 8 (see Chapter 3) and was unusual in that the subject needed resuscitation from profound hypoglycaemia during the test.

After an overnight fast, at time 0, a bolus of glucose (0.5g/kg body weight) was given. Blood for glucose, insulin, glucagon, cortisol and growth hormone levels was drawn at set times relative to the glucose bolus (predose, 15, 30, 45, 60, 90 & 120 minutes post dose). For further details of the test see case 8 in Chapter 3.

c) Short Synacthen test A single intramuscular injection of synthetic ACTH analogue (Synacthen, Ciba Laboratories, UK) was given at time 0. Blood for serum cortisol measurement (Framos RIA, Pharmacia, UK) was drawn predose and at 30 and 60 minutes post dose.

Interpretation A rise in serum cortisol of $\geq$220nmol/l above the predose level with a peak level of at least 500nmol/l was taken as normal.

All samples were assayed in the laboratories of the Department of Biochemical Medicine, Ninewells Hospital and Medical School, Dundee.

2.2 ANIMALS

All animal experiments were carried out under the auspices of a Home Office Project Licence to carry out a Programme of Scientific Procedures on Living Animals granted to Dr. Ann Burchell, Lister Institute Research Fellow, Ninewells Hospital and Medical School, Dundee and a Personal Licence to carry out Regulated Procedures on living animals granted to me. At all times the terms of the 1986 Animals (Scientific Procedures) Act were diligently adhered to.

The rats used to study the hepatic glucose-6-phosphatase system and to purify the postulated microsomal glucose-6-phosphate transport protein were adult male Wistar rats from the colony inbred and cared for in the University Animal Unit, Ninewells Hospital and Medical School, Dundee. An adult female Cheviot sheep was used to raise antibodies to the postulated hepatic microsomal glucose-6-phosphate transport protein (T1).

All the laboratory animals were maintained under standard conditions of temperature and relative humidity with a 12 hour light/dark cycle. They had free access to laboratory animal chow (unless specifically fasted for 16 hours prior to death) and tap water.

2.2.1 Treatment of laboratory animals.

The hepatic microsomal glucose-6-phosphatase system was studied under different metabolic conditions in the rats. These metabolic conditions were achieved as follows.

a) Fed animals These animals had free access to food and water up until death.

b) Starved animals These animals were denied access to food for 16 hours prior to death. They had free access to water at all times.

c) Diabetic animals To induce diabetes the animals were sedated with ether and then given a single tail vein injection of 75 mg/kg body weight streptozotocin in citrate buffered to pH 4.5. Control animals were injected with buffer only. The animals then had free access to food and water for 72 hours at which time they were killed. The development of diabetes was confirmed in the test animals by the measurement of a blood glucose >13 mmol/l using commercially available blood glucose testing strips (BM 1-44 glycaemie sticks, Boehringer, UK).

After death whole livers were removed immediately by dissection and placed into ice-cold 0.25M sucrose/5mM HEPES buffer, pH 7.4. Microsomes were prepared from the livers immediately (see Microsomal preparation 2.5).

2.3 CHEMICALS

Most reagents were of analytical grade or better, depending on availability. Several reagents were especially used, either for their composition or purity, and these are listed below.

The monosodium salt of glucose-6-phosphate, acrylamide, N'N'-BIS methyleneacrylamide, unstained molecular mass markers for SDS-PAGE, Cocktail T (scintillant) and Folin Ciocalteau's Reagent were from BDH Chemicals, Poole, UK.

The monopotassium salt of glucose-6-phosphate, mannose-6-phosphate (disodium salt), amiloride hydrochloride (3,5-diamino-6-chloro-N-(diaminomethylene)pyrazine carboxamide), histone 2A, cacodylic acid (recrystallised from 95% ethanol after Arion & Wallin 1973), streptozotocin (mixed isomers), prestained molecular mass standards for SDS/polyacrylamide gel electrophoresis, 4-Chloro-1-naphthol and Lymphoprep (ficoll + sodium diatrizoate, density 1.077) were all from Sigma Chemicals, Poole, UK.

The protease inhibitor cocktail used to thaw the fast frozen gut mucosa specimens in (see 2.5.1) was from Boehringer Mannheim Biochemica, Germany. Other protease inhibitors were from Sigma Chemicals, UK. The nitrocellulose used for immunoblotting and transport assays (0.45μm pore size) was from Schleicher and Schuell, Germany. Lubrol 12A-9 was from ICI Organics Division, Manchester, UK. All Sepharose used to prepare affinity columns was from Pharmacia, Uppsala, Sweden as were pre-poured Sephadex G-25 M columns.

[U-^{14}C] Glucose-6-phosphate (monosodium salt), ^{22}NaCl, biotin-linked anti-sheep IgG and streptavidin linked horseradish peroxidase were all from Amersham International, Amersham, UK.

^{14}C 1-naphthol and UDPGA were a kind gift from Professor Brian Burchell, Department of Biochemical Medicine, University of Dundee.

2.4. EQUIPMENT

Pipettes and glassware were standard laboratory items. MSD high speed centrifuges, Kontron Ultracentrifuge and Eppendorf benchtop centrifuges were used. SDS-PAGE was carried out using Hoeffer gel electrophoresis equipment. Dot blots and transport assays were performed using Bio-Rad dot blot apparatus. Electrophoretic transfer of proteins onto nitrocellulose from gels was carried out using an LKB Multiphore II semi-dry blotting system. Scintillation counting was performed on Packard 2200 CA (^{14} C) and LKB (^{22}Na) machines. A Philips PU 8620 spectrophotometer containing an autocell and remote sampler was used for all measures of absorbance.

Fast Pressurised Liquid Chromatography (FPLC) was carried out using a system from Pharmacia, Uppsala, Sweden.

2.5 MICROSOMAL PREPARATION

Microsomes were isolated from all animal and human samples wherever possible for study of the glucose-6-phosphatase system. With small tissue samples this was not always possible, and these are stated in the relevant parts of the text: in these instances studies were carried out on the 12,000 xg supernatant from the first centrifugation step described below.

Stock solution: 0.25M sucrose/ 5mM HEPES (SH) pH 7.4

Procedure Samples were homogenised fresh and unfrozen by hand or (for larger samples such as whole rat livers) using a Griffin powered homogeniser in Jencons teflon/glass homogenisers at 4°C in 9 parts (w/v) ice-cold SH pH 7.4. The homogenate was centrifuged at 12,000 xg for 6 minutes at 4°C. The resulting supernatant was then centrifuged at 105,000 xg for 1 hour at 4°C. The microsomal pellet was resuspended in 1.0 ml per gram wet tissue weight SH pH 7.4 by hand in a teflon/glass homogeniser. All samples were assayed fresh, any which were kept were stored at -70°C and used within 3 months of preparation.

2.5.1 Intestinal mucosa samples

Strict conditions were imposed on these samples to prevent proteolysis by intestinal enzyme systems. A different technique was used to handle the samples for phosphatase activity measurement where it was necessary to preserve enzyme activity from that used to handle the samples for immunoblotting where enzyme activity is unnecessary.

Samples for determination of glucose-6-phosphatase and non-specific phosphatase activity were placed immediately into ice-cold SH buffer containing the protease inhibitor cocktail described in Table 2.1 above. The mucosal samples were homogenised immediately by hand in a teflon/glass, ice-cold homogeniser. The homogenate was made to a volume equal to 10 times the original wet weight and centrifuged for 1 minute at 4,000 xg. The supernatant was assayed fresh for both glucose-6-phosphatase and non-specific phosphatase activities as described elsewhere (2.6.1 (a) & (e) and 4.1).

Samples of intestinal mucosa for immunoblotting and staining were frozen immediately after taking in liquid nitrogen. The frozen samples were stored at -70°C and all processed together. The samples were thawed in SH buffer pH 7.4 containing the protease inhibitor cocktail shown in Table 2.2 and homogenised by hand to a final volume 10 times the wet weight of tissue.

Table 2.2

Protease inhibitor cocktail used to thaw and homogenise intestinal mucosa samples. These inhibitors form a commercially available kit (Boehringer-Mannheim, London, UK).

Protease inhibitor	Concentration	Diluent
Antipain dihydrochloride	50μg/ml	water
APMSF*	40μg/ml	water
Aprotinin	10μg/ml	water
Bestatin	40μg/ml	HCl
Chymostatin	100μg/ml	acetic acid
E-64	0.5mg/ml	water
EDTA-Na_2	0.5mg/ml	water
Leupeptin	0.5μg/ml	water
Pepstatin	0.7μg/ml	methanol
Phosphoramidon	330μg/ml	water

*APMSF = (4-amidinophenyl)-methanesulphonyl fluoride

The homogenate was centrifuged for 1 minute at 4,000 xg and the supernatant mixed immediately into a boiling mixture of 200μl sample buffer (see 2.9.2) and 100μl of mercaptoethanol. The whole was boiled for 10 minutes and then frozen at -20°C before being separated by polyacrylamide gel electrophoresis.

2.5.2 Separation of neutrophil polymorphonuclear cells from blood. Boyum 1968 and Ferrante and Thong 1980.

Blood samples were collected as described above. The cell components were separated from the blood immediately.

Stock solutions:

5.7g/dl ficoll + 9g/dl sodium diatrizoate SG 1077
(Lymphoprep, Sigma Chemicals)

Hank's buffered saline pH 7.4 :

0.8% NaCl, 0.04% KCl, 0.005% NaH_2PO_4

0.006% KH_2PO_4, 0.01% $MgSO_4.7H_2O$

0.014% $CaCl_2$, 0.1% glucose

0.01% $MgCl_2$, 0.035% sodium bicarbonate

6% Dextran 70

1.8% sodium chloride

Protease inhibitors-several combinations were tried.
(See 4.2)

a) 1mg/ml 1,10-phenanthroline+1mg/ml PMSF

b) 0.02% PMSF + 0.1mM pepstatin A
+ 10Mm 1,10-phenanthroline+0.01mg/ml
Trypsin-chymotrypsin inhibitor
± 400KIU/ml aprotonin

c) 0.02% sodium azide + 2mM PMSF

Procedure The 10ml blood/anticoagulant was mixed with 5ml Dextran 70 by inversion and then left at room temperature for 90 minutes to allow the red cells to separate out under gravity. The leucocyte-rich top layer was pipetted off and layered onto 5ml Lymphoprep in a conical plastic tube. After centrifuging at 200 xg at room temperature the supernatant was discarded. 5ml distilled water was added to lyse remaining red cells in the pellet followed by 5ml 1.8% sodium chloride 20 seconds later. This was respun at 100 xg for 5 minutes and the resulting pellet washed in Hank's buffered saline containing protease inhibitors. After a final spin at 100 xg for 5 minutes the pellet, comprising neutrophil polymorphonuclear lymphocytes, was resuspended in 0.5ml Hank's containing protease inhibitors. The

number and composition of cells in the suspension was evaluated using a Coulter T540 cell counter.

2.5.3 Disruption of neutrophils

Several methods were tried to disrupt the neutrophils (PMNs) which were isolated as in 2.5.2 at the same time trying to prevent proteolysis of the organelles prior to testing for the presence of glucose-6-phosphatase.

a) Detergent The separated cells were mixed with 0.01% lubrol and frozen at -70°C. The treated cells were thawed in Hank's + protease inhibitors before being dropped into boiling sample buffer and mercaptoethanol (2.9.2).

b) Freeze-thawing Aliquots of isolated PMNs were frozen at -70°C ± protease inhibitors and thawed at 30°C before use

c) Homogenisation Aliquots of separated cells were homogenised with a mechanical homogeniser.

The effects of these treatments is described in Chapter 4.2

2.6 ASSAYS

2.6.1 Colourimetric assays

The assays of glucose-6-phosphatase, mannose-6-phosphatase and pyrophosphatase activity were performed after the methods described by Burchell *et al* 1988a with the addition of histone 2A as described by Blair and Burchell 1988. All three assays depend upon measuring the amount of inorganic phosphate (P_i) produced by incubating microsomes with appropriate substrates (modified from Ames 1966). The P_i released during the incubation reacts with the stopping reagent to produce a blue reduced phosphomolybdate complex the absorbance of which measured at 820 nm is directly proportional to the concentration of P_i (μmol/l) in the solution.

In all the above assays 0.1 M HEPES was used in place of cacodylate (as originally used by Burchell *et al* 1988a) as the buffer, and substrate concentrations were as indicated below.

Stock solutions:

0.3M glucose-6-phosphate (G6P) pH 6.5
12.5mM mannose-6-phosphate(M6P) pH 6.5
0.3M sodium pyrophosphate (PP) pH 6.0
0.1M HEPES pH 6.5
0.1M HEPES pH 6.0
0.1M EDTA pH 6.5
0.1M EDTA pH 6.0
0.01% histone 2A

a) Glucose-6-phosphate substrates: all contained 2 ml stock HEPES pH 6.5 and 0.25 ml stock EDTA pH 6.5.

Final [G6P](mM)	Vol G6P stock (μl)	Vol. H_2O (ml)
1.0	42	7.7
1.4	59	7.7
2.0	84	7.7
2.6	108	7.6
5.0	208	7.6
30	1250	6.5

b) Mannose-6-phosphate substrates: all contained 1ml mannose-6-phosphate (M6P), 2ml HEPES pH 6.5 and 0.25ml EDTA pH 6.5.

c) Pyrophosphate substrates: all contained 2ml stock HEPES pH 6.0 and 0.25ml stock EDTA pH 6.0

Final [PP](mM)	Vol. PP stock(μl)	Vol H_2O (ml)
0.5	21	7.7
1.0	42	7.7
1.4	59	7.7
2.0	84	7.7
2.6	108	7.6
5.0	208	7.6

Substrates with the same G6P, M6P and PP concentrations were made containing 1 ml stock histone 2A, and correspondingly, 1ml less water.

All substrates were stored at -20°C and when thawed were kept on ice. Substrates were discarded if the blank replicate in the assay showed increased absorbance at 820nm as this implied non-enzymatic degradation of the glucose-6-phosphate substrate.

Stopping reagent:

6 parts 0.42% ammonium molybdate in 0.5N H_2SO_4

2 parts 10% sodium dodecyl sulphate

1 part 10% ascorbic acid (made fresh daily)

Procedure The basic assay was identical for all three substrates. 80μl substrate at each concentration was incubated in triplicate (two active and one blank). 20μl of sample (diluted to approximately 2 mg/ml protein in 0.25M sucrose/ 5mM HEPES pH 7.4) was added to the two 'active' tubes and incubated for exactly 10 minutes.

900μl of stopping reagent was then added to all tubes. 20μl of diluted sample was then added to the 'blank ' tubes. All tubes were then incubated to allow colour production before absorbance at 820 nm (OD_{820}) was measured. The mean of the two replicates was taken and the blank value at the relevant substrate concentration subtracted from this mean to calculate the kinetic constants.

Glucose-6-phosphatase and mannose-6-phosphatase assays were performed at 30°C and the colour production at 46°C for 20 minutes (the conditions which allow maximum colour production).

Pyrophosphatase assays were performed at 30°C and the colour production at 30°C for exactly 10 minutes (at 46°C pyrophosphate is acid labile and this significantly increases the OD_{820} blank values). These conditions allowed half-maximal colour development but this was offset by the fact that two P_i molecules were produced, but did mean that a new standard curve had to be performed with each assay to allow for small variations in time or temperature.

After calculation of specific activity (see "d) Phosphate standard curve" below) kinetic constants for glucose-6-phosphatase and pyrophosphatase activity in both intact and disrupted microsomes were calculated from slopes and intercepts of Lineweaver-Burk plots using a BBC computer programme of non-linear regression analysis based upon Colquhon 1971.

From the mannose-6-phosphatase activity it is possible to calculate the "intactness" of a microsomal preparation using the equation:

$$\text{Intactness (\%)} = \frac{\text{s.a.(+h)} - \text{s.a(-h)}}{\text{s.a.(+h)}} \times 100$$

Where s.a. is specific activity and h is histone 2A.

(For explanation of intactness please see Chapter 1).

d) Phosphate standard curve

In order to convert OD_{820} readings into specific phosphatase activity a factor based upon a phosphate standard curve had to be derived.

Stock solution: 1.0mM soluble phosphate (eg NaH_2PO_4)

Stopping reagent: See above.

Procedure Volumes of stock phosphate (1-100μl) were made up to 100μl with water in triplicate. 900μl stopping reagent was added at 10 second intervals and incubated at 30°C for 10 minutes. The standards for glucose-6-phosphatase activity were then incubated at 42°C for 20 minutes and those for pyrophosphatase at 30°C for 10 minutes before the OD_{820} was measured. The standard curve plot was OD_{820} against volume (μl) of stock phosphate added. The correction factor was calculated as follows:

$$\text{Factor}^{-1} = (\mu\text{mol of phosphate to give } OD_{820} = 1.0) \times 5$$

For glucose-6-phosphatase the factor was approximately 4.8; for pyrophosphatase it was 2.3 - the difference between the two reflecting the fact that the pyrophosphatase reaction only took the colour reaction to approximately half completion.

Once the factor had been determined the specific activity (in μmol P_i produced/minute/mg microsomal protein added) of the components of the enzyme system could be calculated from the equation:

$$\text{Specific activity} = \frac{(\text{mean } OD_{820} - \text{blank}) \times \text{Dilution of sample}}{\text{Factor} \times \text{protein concentration in assay}}$$

e) Non-specific phosphatase activity: Burchell and Burchell 1980.

The measurement of non-specific phosphatase activity in samples is vital to the correct assessment of glucose-6-phosphatase, pyrophosphatase and mannose-6-phosphatase activity. The method used relies on the fact that the hydrolytic

component of the glucose-6-phosphatase system is inactivated by incubation at pH 5 and 37°C.

The assay method was exactly as described for glucose-6-phosphatase above, however the samples were preincubated with 2.5% sodium acetate pH 5 (1 part sodium acetate, 20 parts sample) and incubated at 37°C for 15 minutes. 400 times the sample volume of 0.1 M HEPES pH 6.5 was then added and the treated sample assayed as described above. The calculation of non-specific phosphatase activity was also as described above, the correction factor being calculated in the same fashion (Factor = 2 approximately).

In all rat liver microsomal preparations the proportion of non-specific phosphatase activity was <3% of the total phosphatase activity and is not commented upon again.

f) Protein concentrations: Peterson 1977

Stock solutions:	0.8N sodium hydroxide (NaOH)
	10% sodium dodecylsulphate (SDS)
	20% sodium carbonate, 0.1% copper sulphate & 0.2% potassium tartrate
	Folin-Ciocalteau's phenol reagent
Working solutions:	0.15% deoxycholic acid (DOC)
	72% trichloroacetic acid (TCA)
	0.1% bovine serum albumin (BSA)
	Reagent A: equal parts of copper-tartrate-carbonate, NaOH, SDS and water (made fresh daily)
	Reagent B: 1 part Folin-Ciocalteau and four parts water (made fresh daily)

Procedure The sample (approximately 20μg protein) was diluted to 1ml with water. Each sample was assayed in duplicate and a blank was performed with each assay. 0.1ml DOC was added and the tubes incubated at room temperature for 10 minutes before 0.1ml TCA was added. The samples were centrifuged at 3,000 xg for 10 minutes and the supernatant discarded. Each pellet was mixed into 0.4 ml reagent A and 0.4 ml water. After standing for 10 minutes at room temperature 0.2 ml reagent B was added. The OD 750nm was read after a further 30 minutes at room temperature.

A standard curve was prepared using BSA (5-50μl of the stock solution) each time an assay was performed to allow for slight differences in solutions and reagents. Standard curves were linear to a concentration of approximately 60μg BSA. The OD 750nm for the sample assays were averaged and the blank subtracted before the protein concentration in mg/ml was read from the standard curve.

g) Protein estimation: Bradford 1976

This rapid method was used to detect the presence of protein in fractions collected from affinity columns. It was not used to quantify protein concentrations.

Stock solution: Brilliant blue G in 125ml methanol
+ 158ml orthophosphoric acid
made up to 500ml with water.

Procedure Equal volumes (50-100μl) of Bradford reagent and sample were mixed in a clear plastic test-tube. If the sample turned blue instantly then this indicated the presence of protein in the sample. All buffers were also tested to ensure they did not give a false-positive reaction.

h) Glycogen concentration: van Handel 1965

Working solutions: 95% ethanol

Anthrone/H_2SO_4 -(150mg anthrone + 38ml conc.H_2SO_4 + 15ml water)

0.04% glucose standard.

Procedure 25μl sample (approximately 20mg/ml protein) was mixed with 25μl water and 60μl ethanol along with a blank (50μl water and no sample) and then centrifuged at 3,000 xg for 8 minutes. The pellet was mixed into 50μl water and 60μl ethanol and re-centrifuged at 3,000 xg for 8 minutes. The pellet was suspended in 200μl of water and assayed immediately. 10μl of this solution was diluted with 40μl water in triplicate in glass tubes and mixed well with 950μl of ice cold anthrone/H_2SO_4 before being incubated at 90°C for 20 minutes. The OD 620nm was then measured.

A standard curve was prepared using volumes of glucose standard (0-50μl, equivalent to 0-20μg glucose) diluted to 50μl with water before the ethanol extraction step described above. The glucose content of the sample was then read off the standard curve using the mean of the three OD 620nm measurements and the glycogen content of the sample calculated from the following equation:

$$\text{Glycogen concentration (mg/ml)} = \frac{\text{(Glucose content) x Dilution x 160}}{1000}$$

2.6.2 Radionuclide assays

a) Microsomal glucose-6-phosphate transport: Waddell & Burchell 1987a

At first I used the assay described by Waddell and Burchell, but soon modified this to the method described here which was more flexible and, in my hands, more reproducible.

Incubation buffer: 40mM cacodylate pH 6.5
1mM sodium glucose-6-phosphate pH 6.5
5μCi/ml [U^{14}C] glucose-6-phosphate

Procedure Microsomal protein was diluted 100 times in 40mM cacodylate pH 6.5 and 900μl was added to 1.35ml of the incubation mix in a test tube at room temperature, to result in the final concentrations shown above. At the addition of the protein to the incubation mix the clock was started. At the required times (from 15 seconds to 5 minutes) 250μl of the reaction mixture was pipetted into a different well of a Bio-Rad dot blot and sucked rapidly (within 6 seconds) through a sheet of nitrocellulose which had been well wetted with 40mM cacodylate pH 6.5. At the end of the assay the nitrocellulose was dried under an infra-red lamp and then cut into 0.5 x 0.5 cm squares with the (visible) microsomal protein at the centre. Each square was counted in sequence thus allowing the time points to be identified. All assays were performed in triplicate.

A blank assay was performed using microsomes which had been disrupted by pre-incubation with lubrol (5 parts microsomal protein : 4 parts 40mM cacodylate pH 6.5 : 1 part 2% lubrol mixed and incubated on ice for 30 minutes). All microsomes treated with lubrol were assayed with low K_m mannose-6-phosphate to confirm the lack of integrity of the microsomal membrane.

250μl of incubation mix containing no microsomal protein was also passed through the nitrocellulose filter - the level of this buffer blank was always $<2\%$ of the minimum value in the reaction mix, and was therefore ignored.

The squares of nitrocellulose were placed into vials with 4.5ml scintillant (Cocktail T) and the ^{14}C trapped on the filter was counted (for 10 minutes and averaged). As well as squares of nitrocellulose, 25μl of incubation mix + microsomes was also counted - the value obtained was multiplied by 10 to give a value for "total counts".

The capacity for microsomal glucose-6-phosphate transport was calculated from the formula:

$$\text{Capacity for G6P transport (nmolesG6P/mg protein)} = \frac{\text{Retained counts - Blank counts}}{\text{Total counts}} \times F$$

$$\text{Where } F = \frac{\text{nmol G6P in incubation mix}}{\text{mg microsomal protein on each piece of filter}}$$

b) Microsomal sodium transport

Influx: The assay for glucose-6-phosphate transport was modified so that the incubation mixtures contained 1μCi/ml ^{22}NaCl in place of the ^{14}C G6P. The assay procedure was identical to that described for glucose-6-phosphate transport. The pieces of nitrocellulose filter were counted on an LKB gamma counter.

Efflux: This assay required that ^{22}NaCl be incorporated into microsomes and the efflux of the ^{22}Na be followed. Previously prepared low metal ion microsomes (see Chapter 5 for details) were centrifuged at 105,000 xg for 1 hour. The pellet was resuspended in low-sodium SH pH 7.4 containing 10mM NaCl plus 12.5μCi/ml ^{22}NaCl and frozen at -70°C. The treated microsomes were thawed slowly on ice, diluted in 9 parts low sodium cacodylate and added to an incubation mix containing 40mM low sodium cacodylate $\pm$ 1mM potassium glucose-6-phosphate. At the indicated time points (15 seconds - 10 minutes) 250μl was pipetted into a well of the dot blot apparatus and sucked through a presoaked nitrocellulose filter which was then processed as already described.

c) 1-Naphthol assay: Otani *et al* 1976

This assay was used as an alternative method of measuring microsomal membrane integrity (Burchell & Coughtrie 1989). The assay measures the rate of glucuronidation of 1-naphthol with its co-substrate UDPGA. The enzymatic

glucuronidation of 1-naphthol is carried out in the lumen of the endoplasmic reticulum, and is, therefore only expressed in disrupted microsomes.

Stock solutions:

100mM Tris/maleate 5mM $MgCl_2$ pH 7.4

5mM 1-naphthol dissolved in 3% DMSO

4μCi/ml 1-[1-^{14}C]naphthol, dissolved in methanol

20mM UDPGA dissolved in AIK (Alkaline Isotonic KCl - 154mM KCl,brought to pH 7.4 with solid KHCO3)

600mM TCA/400mM glycine, pH 2.2 with solid NaOH

Procedure 30μl of protein (50-150μg) was mixed with 10μl lubrol and incubated on ice for 30 minutes. 90μl Tris/maleate buffer, 20μl 1-naphthol, 10μl 1-[1-^{14}C]naphthol were added and preincubated at 30°C for 2 minutes before the reaction was started by the addition of 40μl UDPGA. Each assay was performed in duplicate and with a blank in which AIK was substituted for UDPGA. After exactly 10 minutes at 30°C the reaction was stopped by the addition of 200μl ice-cold TCA/glycine. Unconjugated 1-naphthol was extracted into chloroform (3ml) by mixing for 4 minutes and then centrifugation for 4 minutes at 2,000 xg. 200μl of the aqueous phase was added to 2.5ml Cocktail T (scintillant). The 1-[1-^{14}C]naphthyl glucuronide was counted for 1 minute. An aliquot of the incubation mix was also counted to provide "total counts".

The specific activity of 1-naphthol glucuronidation was calculated from the equation:

$$\text{Specific activity 1-naphtol GT (nmol 1-napthol/min/mg protein)} = \frac{\dfrac{(\text{Test counts} - \text{blank counts})}{\text{Total counts}} \times 200}{\text{Incubation time(mins)} \times \text{protein (mg)}}$$

2.7 PROTEIN PURIFICATION

Affinity chromatography was the method used to attempt the purification of the rat hepatic microsomal glucose-6-phosphate transport protein. In affinity chromatography a ligand which is known to bind with the protein being sought is linked covalently to a matrix - in all cases Sepharose (Pharmacia, Sweden) was used. Once the ligand has bound, any sites on the matrix which have not bound ligand have to be inactivated (by washing at high/low pH) or blocked (using ethanolamine) to reduce non-specific protein binding to the matrix. The prepared column then has to be protected from chemical and microbial attack (the antimicrobial sodium azide was loaded onto the columns which were kept at 4°C) and from light-induced damage.

A membrane-bound protein (such as the microsomal glucose-6-phosphate transporter) has to be, as far as possible, separated from its membrane, while at the same time trying to maintain enough of the structural integrity of the protein to allow it to be recognised by the ligand. This process here has been called solubilisation. The solubilised protein is run onto the column and left to incubate for sufficient time to allow binding of ligand and protein. Excess/unwanted protein is then washed of with buffer before an eluent is run through the column. An eluent is usually a compound which has more affinity for the isolated protein than the ligand, or which can break the bonding between ligand and protein.

Fractions from the column are collected (from when the eluent was first loaded onto the column) and stored.

Stock solutions	Tris-buffered saline (TBS):10mM Tris/HCl pH 7.4, 0.9% saline
	TBS + 0.002% sodium azide
	0.1M sodium bicarbonate pH 8.3
	0.1M sodium acetate pH 4
	1M ethanolamine pH 9
	2% lubrol 12A-9

2.7.1 Glucose-6-phosphate.

The binding of the physiological ligand glucose-6-phosphate to the transport protein is unlikely to be strong enough to make affinity chromatography possible, but was tried in case the binding was stronger than expected.

Preparation of the column. Epoxysepharose 6B was swollen in a large excess of water at 46°C before being incubated and shaken constantly at room temperature overnight with 40mM glucose-6-phosphate pH 8.3.

The Sepharose was washed on sintered glass with a large excess of water, 0.1M sodium bicarbonate pH 8 and 0.1M sodium acetate pH 4 in rotation, each wash being used twice. The Sepharose was then incubated with 1M ethanolamine at 46°C for 4 hours before being washed with water and loaded into the barrel of a 5ml syringe (with glass wool in the tip).

The completed column (approximately 3ml) was washed through with 20ml Tris-buffered-saline (TBS) and stored at 4°C in the dark in TBS containing 0.002% sodium azide.

Solubilisation of microsomes. 5ml of microsomes prepared from the livers of streptozotocin-induced diabetic rats were mixed with 0.5ml agarose Concanavalin A at 4°C for 10 minutes and then centrifuged at 2,500 xg for 10 minutes. The pellet (remnant cell membrane) was discarded and the supernatant mixed with 0.5%

Triton X in the ratio 8 Triton X : 5 supernatant (v/v). This mixture was centrifuged at 105,000 xg for 1 hour at -4°C and the resulting pellet resuspended in 8% Triton X to a final volume of 5ml using a hand-held glass homogeniser. This homogenate was frozen and stored overnight at -70°C; the next day it was thawed and centrifuged at 105,000 xg for 1 hour at 4°C. The supernatant was aliquoted and kept at -70°C.

An alternative method for pretreating microsomes was also used - microsomes from livers of streptozotocin-induced diabetic rats were mixed with SH pH 7.4 and 2% lubrol 12A-9 (ratio v/v 5:4:1 respectively) and incubated on ice for 1 hour before diluting in 19 parts SH pH 7.4 to load onto the column.

2.7.2 Phlorizin

Phlorizin has been shown to be an inhibitor of microsomal glucose-6-phosphate transport (Arion *et al* 1980, Waddell & Burchell 1987a). Although the mechanism of phlorizin binding to T1 is unknown, much is known of its binding to other proteins. However it seemed suitable as a ligand to attempt to isolate the glucose-6-phosphate transport protein.

Preparation of the column. 1.0g Epoxysepharose 6B was swollen in distilled water at 46°C and then washed on scintered glass with a large excess of water. 100mg phlorizin was dissolved in 1ml dimethylformamide - the solution was blue, but turned yellow when the pH was adjusted to the working pH (approximately pH 8) - 3 drops of 10M sodium hydroxide were required. The solution was made to 10ml with 1M sodium chloride in 0.1M sodium bicarbonate pH 8.3.

The swollen, washed Sepharose was incubated at room temperature overnight being continuously shaken. Next morning the solution was brown. The Sepharose was washed with a large excess of, in rotation twice, water containing 10% dimethylformamide, water, 0.1M sodium bicarbonate pH 8.3 and 0.1M sodium

acetate pH 4 then incubated with 1M ethanolamine at 40°C overnight- again being shaken continuously.

The Sepharose was washed with large excesses of water, sodium bicarbonate pH 8.3 and sodium acetate pH 4 in rotation, each one twice, and loaded into the barrel of a 5ml syringe with glass wool in the tip, washed with 50ml TBS and stored at 4°C in the dark loaded with TBS containing 0.002% sodium azide. The final volume of column was approximately 3ml.

2.7.3 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS)

DIDS is a reversible inhibitor of microsomal glucose-6-phosphate transport (Zoccoli & Karnovsky 1980 and Arion *et al* 1980b) and was therefore an obvious choice to try and isolate the microsomal glucose-6-phosphate transport protein.

Preparation of the column. Epoxysepharose 6B was swollen in water at 46°C before being washed on scintered glass with a large excess of fresh water. The Sepharose was then incubated with 5mg/ml DIDS at 40°C overnight while being continuously shaken (the sepharose turned pale yellow), washed with excess water and incubated over the next night with 1M ethanolamine at 42°C. After another wash with excess water the sepharose was loaded into a 5ml syringe with a small glass wool plug in, or into an XK 16/20 column for use on a fast, pressurised liquid chromatography (FPLC) system. The columns were then washed with TBS (volume 10x that of the column) before being stored at 4°C in the dark (DIDS is light-sensitive) containing TBS and 0.002% sodium azide.

Running the columns. All the columns described above were run in the same way. The column was brought to room temperature and washed with 50ml TBS + 0.2% lubrol before detergent-treated microsomes from streptozotocin-induced diabetic rats (see above), diluted in 19 parts TBS, were loaded. The column + microsomes were incubated at room temperature for 1 hour and then washed with 20ml TBS + 0.2%

lubrol. The bound protein was eluted with 6ml 1M sodium chloride pH 9 and the column then washed with 50ml TBS + 0.2% lubrol. 0.25ml fractions were collected, tested for the presence of protein with the Bradford reagent and then stored at -20°C.

The DIDS/sepharose column for the FPLC system was run under exactly the same conditions, except all the washes were automatic, the column was much larger (volume 20ml compared to 3ml) so the amount of protein loaded was greater, and the presence of protein in the fractions was shown by a peak of infra-red absorption on a chart recording.

2.7.4. Anti-T1 antibody

Once a protein (the putative glucose-6-phosphate transport protein) had been isolated from the DIDS/Sepharose column and an antibody raised to it in a sheep (see below) one step in trying to characterise the protein using the antibody was to use the latter to try and isolate more of the former by affinity chromatography.

Preparation of the column. CNBr-activated Sepharose 4B was washed and swollen in 1M hydrochloric acid (200ml/g Sepharose). One part anti-T1 IgG from ammonium sulphate treated serum was diluted in 2 parts 0.1M sodium bicarbonate pH 8.3 containing 0.5M sodium chloride and incubated with the CNBr-Sepharose overnight on a rotary mixer at 4°C. The Sepharose was then washed with excess sodium bicarbonate pH 8.3 containing 0.5M sodium chloride and then incubated at 4°C for 16 hours with 1M ethanolamine pH 9. Three washing cycles were then performed, each cycle comprised 0.1M sodium acetate pH 4 and 0.1M Tris pH 8, both containing 0.5M sodium chloride. The Sepharose was then loaded into the barrel of a 5ml syringe, the tip of which contained a small glass wool plug.

The column was stored in the dark at 4°C having been washed with TBS and 0.002% sodium azide.

Solubilisation of microsomes. Microsomes prepared from the livers of streptozotocin-induced diabetic rats were treated as described above. After incubation on ice the microsomal mixture was diluted to 30x its original volume with TBS and the whole volume run through a Sephadex G-25M column (Pharmacia, Sweden). The early fractions from this column contained little lubrol (it runs slower through Sephadex G-25M than protein) and were loaded onto the anti-T1 antibody column.

Running the column. The antibody column was brought to room temperature and washed with 30ml TBS before the microsomal preparation was loaded on and incubated at room temperature for 1 hour. 20ml TBS was then washed through the column followed by elution with fresh 0.1M glycine pH 3. 1ml fractions were collected. The Bradford reagent was of no use as glycine alone turned it blue. The fractions were stored at -20°C. The column was washed with 50ml TBS before storage at 4°C.

All columns were used on several occasions. Between uses they had TBS containing 0.002% sodium azide (apart from the anti-T1 column which had TBS alone on it) loaded onto them and were stored in the dark at 4°C.

The fractions collected from all columns were stored at -20°C before being separated by SDS-polyacrylamide gel electrophoresis (see below). The samples from the DIDS column which were injected into the sheep to produce antibody were concentrated and pooled 4 times using a Speedivac vacuum system. By this method injections of approximately 3mg protein/ml were given to the sheep.

2.8 PREPARATION OF ANTI-T1 IgG

A Cheviot sheep was injected 3 times with a total of approximately 100mg of protein, molecular weight about 66 kDaltons which was isolated by affinity for

DIDS/Sepharose. A month after the final injection a test sample of serum was taken from the sheep and was shown to cross-react with a protein of rat hepatic microsomes by immunostaining (see below). A 200ml sample of serum was then prepared from the sheep.

IgG was purified by ammonium sulphate fractionation (Fahey & Terry 1978 modified by Burchell and Cain 1985).

Procedure Equal volumes of serum and 90% saturated ammonium sulphate were mixed on ice for 15 mins. The mixture was then centrifuged at 5,000 xg for 20 minutes and the pellet resuspended in the minimum possible volume of TBS. (Typical final volume was 190ml from 200ml serum). The resuspended pellet was then dialysed against TBS at 4°C overnight with several changes of dialysate. The resulting semi-purified antibody was aliquoted and stored at -20°C until required. For immunostaining of nitrocellulose-bound proteins a dilution of the anti-T1 IgG of 1:250 was used.

2.9. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

2.9.1. Preparation of gels.

The method of Laemmli 1970 was used to run 7-16% or 7-20% gradient or 10% polyacrylamide gels. The gels were poured between vertical glass plates and electrophoresis was carried out using a Hoeffer gel electrophoresis tank with a Bio-Rad power pack.

Stock solutions :

A: 29.2% acrylamide + 0.8% N'N'BIS methylene acrylamide

B: 18.15% TRIS base pH 8.8

C: 3.0% TRIS base pH 6.8

D: 1.5% ammonium persulphate (fresh daily)

E: 2% sodium dodecyl sulphate

F: N,N,N',N'-Tetramethylethyl--enediamine (TEMED)

Electrode buffer: 3.0g TRIS base
14.9g glycine
1g sodium dodecylsulphate
Made up to 1 litre.

Gels were prepared according to the following recipe:

% Acrylamide	7	10	16	20	4(stack)
Water (ml)	7.7	6.26	3.38	1.45	10
A (ml)	3.38	4.81	7.7	9.65	2.0
B (ml)	1.8	1.8	1.8	1.8	---
C (ml)	---	---	---	---	5.0
D (ml)	0.25	0.25	0.25	0.25	2.0
E (ml)	0.86	0.86	0.86	0.86	0.86
F (μl)	5.0	5.0	5.0	5.0	10.0

Gel solution preparation and pouring of gels was carried out in accordance with the precautions in the Bio-Rad Technical Bulletin No. 1156 (1984).

The solutions A - E were mixed in a beaker. The TEMED was added immediately prior to adding the solutions to a gradient maker (LKB 110ml) for pouring of the gradient gels. Non-gradient gels were poured from a pipette. The glass plates were mounted vertically in a Hoeffer gel system. 0.5 cm of water was layered onto each gel after pouring to prevent inhibition of polymerisation by atmospheric O_2. The

resolving gels were left to set at room temperature overnight before the stacking gel was poured onto the top. The stacking gel was left 20 minutes at room temperature to set. Once set the comb was removed and the wells were filled with electrode buffer.

2.9.2 Preparation of samples

Protein samples for gel electrophoresis were homogenised, denatured and given a negative charge by mixing them thoroughly with mercaptoethanol and sodium dodecylsulphate contained in a solution of TRIS pH 6.8 and glycerol including the dye bromophenol blue (Sample buffer). The mixture was then boiled for 10 minutes before being loaded into the wells of the stacking gel using a gas-tight small volume syringe.

10 - 150 μg of protein were loaded onto gels in volumes from 10 - 200μl. 20μl of both sample buffer and mercaptoethanol were used for all samples unless otherwise stated in the text. Wells which did not contain protein samples were loaded with sample buffer to prevent protein spreading from adjacent lanes.

Standard molecular weight markers were used - either unstained for gels to be directly stained, or prestained for gels which were to be blotted. The unstained molecular weight markers were treated as samples by mixing and boiling with mercaptoethanol and sample buffer. The prestained molecular weight markers were mixed with 8M urea, sample buffer and mercaptoethanol before boiling for 5 minutes. The markers were then aliquoted and stored at -20°C. Before use the aliquot was boiled again for 10 minutes.

The contents of the unstained and prestained molecular weight standards are shown in Table 2.3 (a+b) respectively.

Table 2.3 (a) Unstained molecular weight markers

Protein	Approx. molecular weight
Ovotransferrin	76-78,000
Bovine serum albumin	66,200
Ovalbumin	42,700
Carbonic anhydrase	30,000
Myoglobin	17,200
Cytochrome c	12,300

(b) Prestained molecular weight markers

Protein	Approx. molecular weight
α_2-Macroglobulin	180,000
ß-Galactosidase	116,000
Fructose-6-phosphate kinase	84,000
Pyruvate kinase	58,000
Fumarase	48,500
Lactic dehydrogenase	36,500
Triosephosphate isomerase	26,600

2.9.3 Running of gels

Gels were run using a vertical Hoeffer gel tank, the electrode buffer in top and bottom tanks was as described above. A Bio-Rad power pack was used to give a constant current of 10mA per gel through the stack (about 45 minutes) and 30mA per gel through the main gel. The current was switched off when the dye front was 1-2cm from the bottom of the gel. At all times the system was cooled by running water and the bottom tank was stirred using a magnetic stirrer.

2.10 STAINING AND BLOTTING OF GELS

2.10.1 Protein staining

a) Coomassie blue staining Gels were placed directly into a solution of 14% acetic acid and 10% methanol containing sufficient Coomassie blue dye to make the solution royal blue. The gel was left in the stain overnight and then put into "destain" (14% acetic acid, 10% methanol). The non protein bound blue stain leached out into the destain which needed changed frequently. Eventually the protein bands appeared as blue against a clear background. This method of staining will detect down to 10μg of protein.

b) Silver staining Oakley *et al* 1980 as modified by Morrissey 1981.

This method of staining gels is more sensitive and will detect 10ng of protein. The proteins show up as brown through to black bands depending upon the amount present (darker band means more protein). The gel is washed several times in the following sequence. The volumes of washes are 100ml unless otherwise stated. The times are also minimum times - for all except the developing steps the longer the time of the wash, the better. All solutions were made and used fresh.

Procedure:

1) 14% acetic acid + 10% methanol for 1 hour

2) 10% glutaraldehyde for 30 minutes

3) Water: several large volume washes for a minimum 2 hours, best overnight

4) 5μg/ml dithiothreitol for 30 minutes

5) 0.1% silver nitrate for 30 minutes

6) Water: 250ml for 30 minutes x2

7) Developer (3% sodium carbonate containing 50μl/100ml formaldehyde) - two rapid washes in small volumes.

8) Developer until the desired staining is achieved.

9) 3% citric acid to stop the developing reaction.

10) Store the gel in 0.03% sodium carbonate in the dark to prevent fading.

2.10.2 Blotting of proteins from gels onto nitrocellulose (Western blotting)

Towbin *et al* 1979 as modified by Burnette 1981 and Lin & Kasamatsu 1983.

Stock solution: Blotting buffer = 0.04M glycine,
0.05M Tris,1.3 mM sodium dodecyl sulphate + 200ml 20% methanol
Made up to 1 litre

A sheet of nitrocellulose and 8 pieces of blotting paper were cut to be slightly larger than the gel and soaked in blotting buffer for 30 minutes. The gel, nitrocellulose and blotting paper were arranged as in Figure 2.1 (a). If two gels were blotted simultaneously then the set up of the blotting apparatus was as in Figure 2.1 (b). No more than 2 gels were blotted at the same time. Care was taken not to touch the gel and nitrocellulose with skin or any other source of protein.

2.10.3 Immunostaining of blotted proteins.

The streptavidin-horseradish peroxidase system was used with 4-chloro-1 naphthol as substrate (Domin *et al* 1984). The principals of this method are shown diagrammatically in Figure 2.2.

Stock solutions:
3% bovine serum albumin in TBS
5% skimmed milk solution (Marvel) in TBS
2% Tween 20 (polyoxyethylenesorbitan monolaureate)
TBS ± 2% Tween
Primary antibody - 1:1000 in TBS unless otherwise stated. (Concentration varies with protein being stained).

Anti-IgG - Biotin-linked anti sheep IgG (as primary antibodies raised in sheep). 1:300 in TBS.

Streptavidin - complexed with horseradish peroxidase.1:1000 in TBS.

TRIS/HCl - 50mM TRIS to pH 7.6 with HCl

Substrate -100ml contains (made fresh):

40mg 4-chloro-1-naphthol in 10ml methanol, 90mM Tris/HCl, 100μl hydrogen peroxide

Procedure (all washes are 100ml at room temperature unless otherwise stated: times given are all minimums):

(1) The blot was blocked with successive washes of 3% bovine serum albumin in TBS (37°C, 30 minutes, usually overnight); 5% skimmed milk solution (37°C, 30 minutes) and 2% Tween. The purpose of the block is to bind protein to the nitrocellulose so that the antibodies do not bind non-specifically to all of the membrane.

(2) Wash with TBS + 2% Tween x4 followed by TBS alone x1 (10 minutes each).

(3) Incubate with primary antibody (1 hour).

(4) Wash with TBS + 2% Tween x4 followed by TBS alone x1 (10 minutes each).

(5) Incubate with anti-IgG (15 minutes).

(6) Wash with TBS + 2% Tween x4 followed by TBS alone x1 (10 minutes each).

(7) Incubate with Streptavidin (20 minutes)

(8) Wash with TBS + 2% Tween x1 followed by TBS alone x1 (10 minutes each).

(9) Wash with Tris/HCl (6 minutes).

(10) Incubate with substrate solution (until desired staining produced).

(11) Wash with TRIS/HCl x3 (10-20 minutes each).

(12) Nitrocellulose was then dried under an infra-red light source and stored in the dark in an air-tight packet.

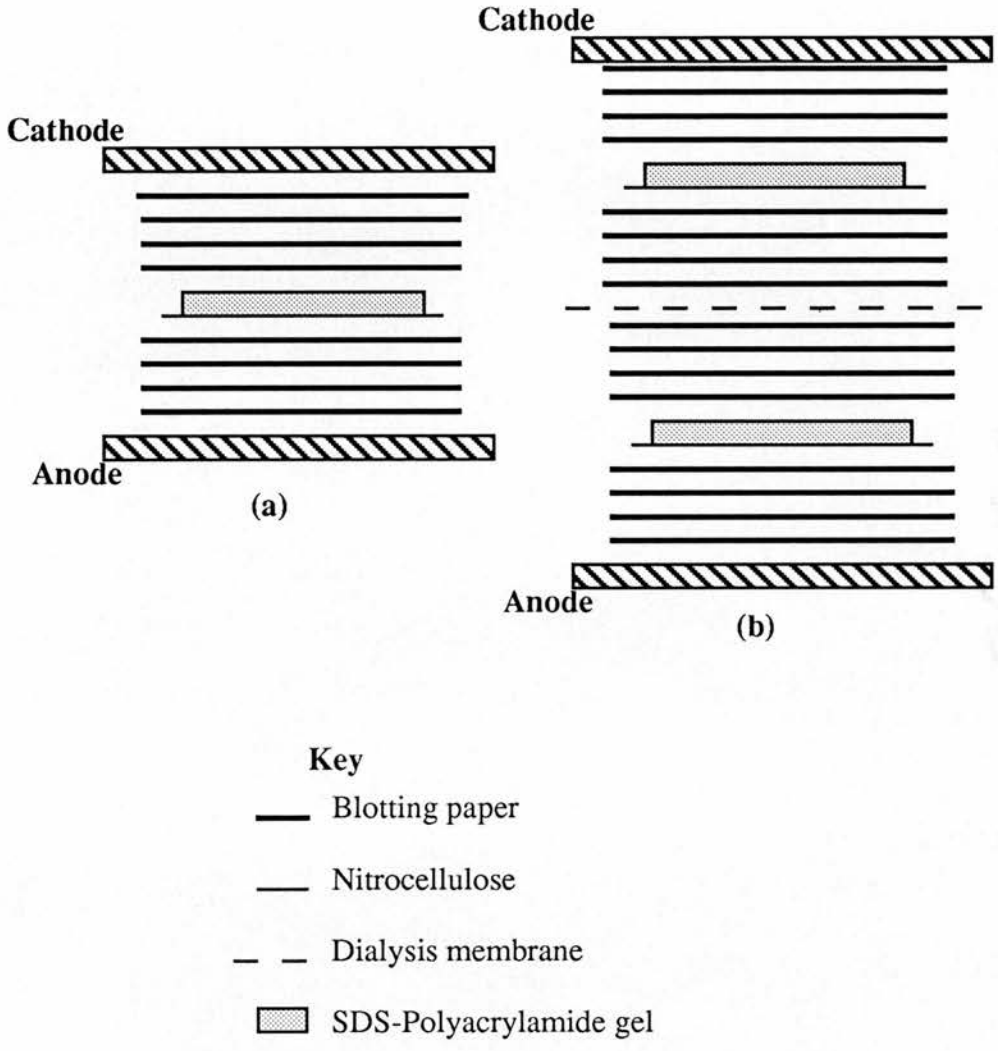


Figure 2.1

Diagrammatic representation of the semi-dry electrophoretic apparatus used to transfer proteins from SDS-polyacrylamide gels to nitrocellulose. Figure (a) shows the technique for transfer from one gel; the simultaneous transfer from 2 gels is shown in (b).

All paper and membranes were thoroughly wetted in electrode buffer (see text) before use.

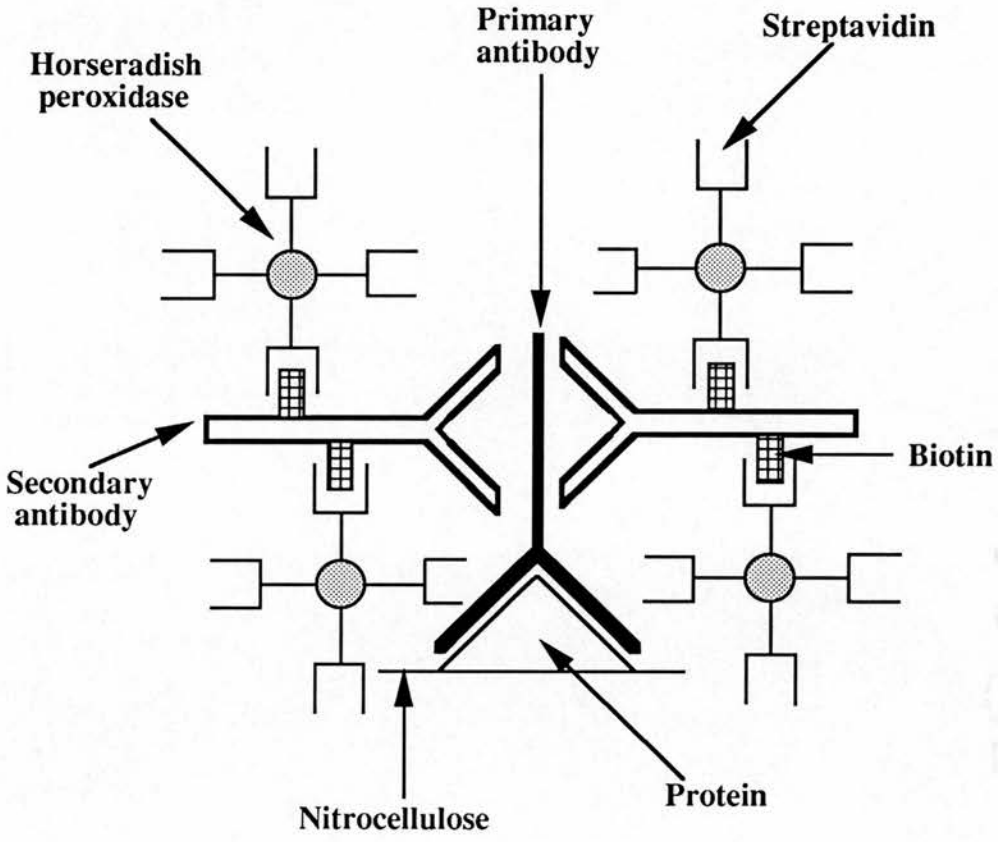


Figure 2.2

Diagrammatic representation of the immunoblot developing technique employed during the work in this thesis. At each step there is an amplification of the signal. 4-Chloro-1-naphthol and hydrogen peroxide were the substrates for the horseradish peroxidase.

This method of immunostaining proteins on membranes was also used for proteins which were directly blotted onto nitrocellulose.

All descriptions given above were the routine methods used. Any variation in assay technique is described in the relevant place in the text.

At all times safety standards and guidelines were closely observed. Before handling radioisotopes I attended a Radioisotopes User course organised by the Department of Medical Physics, Ninewells Hospital and Medical School, Dundee.

CHAPTER THREE

GLUCOSE-6-PHOSPHATASE ABNORMALITIES DIAGNOSED IN ADULTS

One of the major aims of my thesis was to try and link the increase in understanding of the biochemistry of the glucose-6-phosphatase system over the last 10 years or so with clinical problems in adults. The initial idea was to study diabetes mellitus, but as I started my work data was just becoming available on the first adult patients newly diagnosed as having underactivity of hepatic glucose-6-phosphatase and this is where my work started.

Deficiencies in glucose-6-phosphatase activity - the Type 1 glycogen storage diseases (GSDs) - have become increasingly well recognised since the first description of a case in 1952 by Cori & Cori. The recognition of these abnormalities has been made easier over the last few years by improved understanding of the glucose-6-phosphatase system (Arion *et al* 1980b, Burchell *et al* 1988b and Waddell *et al* 1988), improved microassays for components of this system (Waddell *et al* 1987, Burchell *et al* 1988b and Blair & Burchell 1988) and the production of antibodies to specific parts of the glucose-6-phosphatase system (Countaway *et al* 1988 and Waddell *et al* 1988b).

Type 1 GSDs are usually recognised as diseases of infants, and less severely affected children can survive to adulthood (see Moses 1990 and Burchell & Waddell 1991 for recent reviews). There are reports of Type 1 GSD cases being diagnosed in adulthood (Stamm & Webb 1975 and Kuzuya *et al* 1983) but these are noteworthy. In 1987 members of our group presented preliminary data on some of the cases described here (Burchell *et al* 1987) and which I have included here as there are further data to include on these individuals. During my period in Dr. Burchell's

laboratory I was responsible for taking liver biopsies to increase the pool of normal data to include samples from 60 livers from patients who did not have abnormal carbohydrate metabolism. At the time of case 1's terminal admission I was the medical registrar responsible for his care and the first liver biopsy.

The laboratory assays on the biopsy samples taken from these patients were mostly performed by a technician in Dr. Burchell's laboratory (Mrs. Lesley Gibb) who was very experienced at performing these assays on small tissue samples, usually from infants.

Using the new techniques outlined above and with local understanding of glucose-6-phosphatase a total of 8 adult cases of type 1 GSD variants have been recognised in Tayside (plus another 2 cases of partial phosphorylase kinase deficiency described in Appendix C) - six of whom were variants of previously identified type 1 GSDs, and two who showed unrecognised abnormalities of the hepatic glucose-6-phosphatase system.

These cases have all been the subjects of papers in the medical literature, and copies of the published papers can be found in Appendix A. Cases 1, 2, 3, 5 and 6 make up the report by Burchell *et al* 1987, all the cases (plus the two in Appendix C) are the subject of Pears *et al* 1992 (which also includes immunoblot analysis performed by Ian Waddell) and case 8 is the subject of a published case report (Pears *et al* 1991).

Case reports

Case 1

Male, born 19.11.32, normal birth at term and breast fed. Mother had five other pregnancies all producing live infants (no still births or miscarriages), one of which died aged 12 months of 'pneumonia' and another died aged 18 months of 'gastritis'. The other siblings were described as normal. The patient had always been 'backward' mentally, his parents said. There is no subsequent information regarding

his schooling. He was a life-long bachelor who worked for Remploy (a firm who give work-experience to retarded people) until 1971 from when he became unemployed, largely it appears because of chronic ill-health (especially recurrent arthritis).

In July 1940 at age 8 years he was admitted first to hospital dehydrated from vomiting induced by an ear infection. At the time of his admission his parents said that as an infant he had had recurrent spontaneous epistaxes and was regularly constipated. While in hospital he became jaundiced and hepatomegaly was noted (the liver edge was recorded at the umbilicus). The jaundice fluctuated (monitored clinically and by urinalysis) and was associated with periodic delirium. At the onset of jaundice, peripheral blood agranulocytosis was seen but subsequent blood films during this illness were normal. He had been seen playing in open drains before the illness. A diagnosis of leptospirosis was excluded by a normal lumbar puncture with no direct evidence of leptospira in blood or urine and negative serum agglutination tests. The final diagnosis given for the jaundice was 'subacute yellow atrophy' although a liver biopsy was not performed.

In 1965 aged 33 years he was investigated for mild rectal bleeding thought to be due to a small anal fissure. Hepatomegaly (described as 3-4 finger breadth) was again noted. No bilirubin was detected in his serum, total serum protein and albumin were measured as 83 and 50 g/l respectively and serum protein electrophoresis is described as being within normal limits. Detailed coagulation studies were performed - Lee and White whole blood clotting times were 6, 6.5 and 6.5 minutes; Duke bleeding time was 2.5 minutes; one-stage prothrombin time was 14 seconds (control 15 seconds); thromboplastin generation test and peripheral blood films were normal. The only abnormality was a slightly positive Hess test. He was clinically hypogonadal - small testes, female distribution of body hair, poor beard growth (he shaved three times a week) and was noted to be of short height (his adult height was

5ft 3in, extended arm span 5ft 5in). Buccal smears were examined and showed no sex chromatin.

That same year he developed chronic paronychia of the left great toe due to an ingrowing nail which was excised. The infection and ingrowing nail recurred requiring nail bed ablation.

In 1966 at age 34 years he was seen with a 6 week history of painful swelling of his right elbow which had followed minor trauma. He also gave a two week history of mildly painful swelling of his right foot. Clinical examination showed arthritis of the right elbow and right first metatarsophalangeal joints. Routine serum urea, electrolytes and liver biochemistry were normal. Blood film showed mild iron-deficient picture - serum iron was low at 7.2 μmol/l. The erythrocyte sedimentation rate in the first hour was 'elevated' (figures are not recorded). RA latex test was initially strongly positive, but was negative two weeks later. Serum uric acid was raised at 0.7 mmol/l falling with colchicine therapy to 0.15 mmol/l. A glucose tolerance test (as a test for malabsorption) was performed - Table 3.1. The arthritis settled with rest and analgesia (regular paracetamol) and he was given oral iron replacement therapy.

In 1967 he was seen by at a psychiatric out-patient clinic suffering from depression. No drug therapy was given.

In 1970, aged 38 years, he had a further episode of polyarthritis affecting the metatarsophalangeal joints of both great toes and the tarsal joints of the right foot. There was no clinical or radiological evidence of rheumatoid disease. Hepatomegaly almost to the umbilicus and small testes were again noted along with a left hydrocoele. Other examination was unremarkable. ESR was raised at 83mm in the first hour. Serum iron was again slightly low, but haemoglobin was 11.7g/dl and the peripheral blood film was normal. Serum uric acid, on salicylate therapy, was 0.89 mmol/l. Other serum biochemistry including liver function tests (bilirubin undetectable, total protein and albumin 75 and 43 g/l respectively) were normal as

was a bromsulphthalein test (levels at 4, 10, 25 & 45 minutes were 100, 48, 6.5 & 3% respectively). Antinuclear factor, antimitochondrial and antismooth muscle antibodies were not detected in the serum. Radiology of the affected joints showed soft tissue swelling only. Treatment with salicylates was not tolerated because of vomiting (a barium meal at this time showed a little gastro-oesophageal reflux only and no cause for the iron deficiency). The arthritis settled with rest and phenylbutazone. Allopurinol was added once the acute phase was over, the presumptive diagnosis being one of gout. The hydrocoele was aspirated and the aspirate contained epithelial cells only.

In July 1971, aged 38 years, he collapsed unconscious after feeling light-headed, his legs turn to jelly and noticing his vision blur. Physical examination at the time of collapse was unhelpful in determining the cause and the lack of sweating is particularly mentioned. His liver was noted to be one hand's breadth enlarged. Blood glucose shortly after the collapse was 3.9mmol/l. He had painful swelling of his left elbow and the hydrocoele had re-collected (further aspiration again revealed epithelial cells only). He gave a history of further rectal bleeding for which no cause could be found on sigmoidoscopy. He claimed to have recently lost a job due to recurrent nose-bleeds. Further blood clotting studies were performed - prothrombin time, kaolin cephalin clotting time, fibrinogen times with saline and protamine, fibrinogen titres, factor VIII levels, bleeding and whole blood clotting times were all normal. Bone marrow examination was normal. He gave a history of episodic breathlessness on exercise (on occasion he could not climb a flight of stairs) but no exertional chest pain, orthopnoea or PND. His chest was clear to auscultation and chest X-ray and ECG were both normal. This symptom is not mentioned again, nor were any further investigations performed.

A needle liver biopsy was taken in view of the hepatomegaly. Histology was reported as showing marked swelling and widespread fatty change with collagen laid down between hepatocytes and small foci of inflammation. Staining for proamyloid

was negative and for glycogen was normal, although comment is specifically made that the biopsy could represent a glycogen storage disorder and that the high fat levels in the sample could make high glycogen stores appear normal. Overall, however, it was thought that the biopsy was from a large nodule of a macrocirrhotic liver.

A psoriatic-like dermatitis was noted over the extensor surfaces of the elbows and on his hands. No clear history is recorded but a skin biopsy showed patchy peri-vascular infiltration with mononuclear cells and slight hydropic degeneration of the basal cells of the epidermis. The condition appears to have resolved with emulsifying ointment.

Some endocrine investigations were also performed: urinary excretion of 17-OHCS and testosterone were low at 18.4mmol/ and 26.5nmol/l respectively. Urine excretion of HCG was normal at < 1.6 U/24 hours. Serum cortisol was 441.6 mmol/l at 0800 and 184.9mmol/l at 2200 and a short synacthen test was normal (baseline 463.7mmol/l to peak 1162.0 mmol/l). No cause for the collapse was found and he was discharged.

In 1976 aged 44 years he was readmitted with recurrent blackouts. History and examination were unhelpful and investigations showed a normal random blood glucose (3.9mmol/l). No cause for the blackouts was found.

He was followed in out-patients until 1984, during which time he had several episodes of polyarthritis affecting mainly his elbows, knees and first metatarsophalangeal joints associated with marginally raised serum uric acid levels and an ESR of between 20 and 40mm in the first hour. On only one occasion was synovial fluid examined for crystals and it was negative. In 1984 (aged 52 years) the serum gamma glutamyl transferase level was found to be elevated at 55 U/l (upper limit normal = 42 U/l) with a slight increase in serum alkaline phosphatase of 188 u/l (upper limit normal = 120 U/l). These abnormalities persisted.

In 1986 aged 53 years he was admitted to hospital with a six month history of cough productive of white sputum and 3 months of increasing dyspnoea, initially on exertion but latterly at rest. He described episodes of vomiting associated with right upper quadrant abdominal pain. On examination he had air hunger, a tachycardia and normal blood pressure but was pyrexial at 38°C. He had 8-finger breadth, smooth, non-tender hepatomegaly and his chest was clear to auscultation. There was no jaundice but he showed spider naevi, xanthelasmata and gouty tophi.

Initial blood glucose estimation on stick testing (BM 1-44 glycaemie stick, Boehringer, Germany) was 1mmol/l. The rest of his blood biochemistry at presentation showed a metabolic acidosis with a degree of respiratory compensation (an arterial sample showed pH 7.38, pCO_2 15.6mmHg, pO_2 45.1mmHg, bicarbonate 9.3mmol/l, and base excess of -12.1). No salicylate or ethanol was detected in his blood but blood lactate level was very high at 12.5mmol/l (normal < 1.8mmol/l). ESR was 125mm in the first hour, haemoglobin was 10.5g/dl and peripheral total white count was 11.9 x 10^9/l. Blood film showed anisocytotic red cells with some large polychromatic macrocytes and a neutrophil leucocytosis. Prothrombin and kaolin cephalin clotting times were identical to controls. Blood urea was 10.1mmol/l, creatinine 143μmol/l, bilirubin 27μmol/l, serum alkaline phosphatase 2124U/l and aspartate aminotransferase 767IU. Culture of sputum, urine and blood were initially and subsequently negative. Hepatitis B surface antigen was not detected in serum. Chest and plain abdominal radiographs were normal.

Treatment of the hypoglycaemia and dehydration was started with intravenous dextrose and he subsequently required a continuous infusion of 1.5 litres of 10% dextrose per day (2.5g dextrose/kg body weight/day) to maintain a blood glucose level above 4mmol/l. 1.5 litres of 1.4% sodium bicarbonate was infused intravenously per 24 hours for 48 hours by which time the acidosis had corrected.

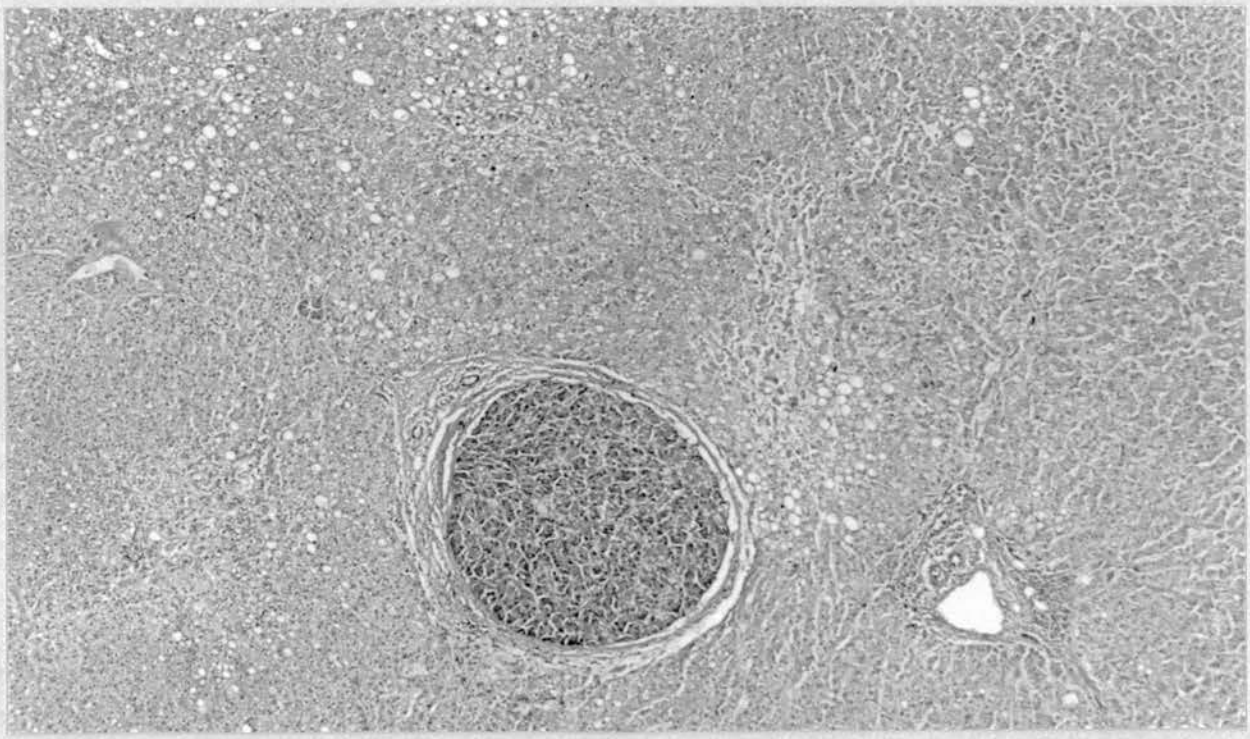
Broad spectrum antibiotic therapy was started and continued despite negative culture results.

Thirty-six hours after admission, he developed oliguric renal failure (despite being normotensive at all times) and gastrointestinal haemorrhage, requiring blood transfusion. He continued to show air hunger despite correction of the metabolic acidosis, and he became drowsy and pyrexial. The haemorrhage stopped and the oliguria improved with infusion of low dose dopamine. Cerebral CT scan was normal and lumbar puncture revealed crystal-clear CSF at an opening pressure of 130mm water, glucose 5.6mmol/l (blood glucose 6.7mmol/l) and protein concentration 370mg/ml. Three white and 5 red blood cells per ml CSF were seen and no organisms were seen or subsequently cultured.

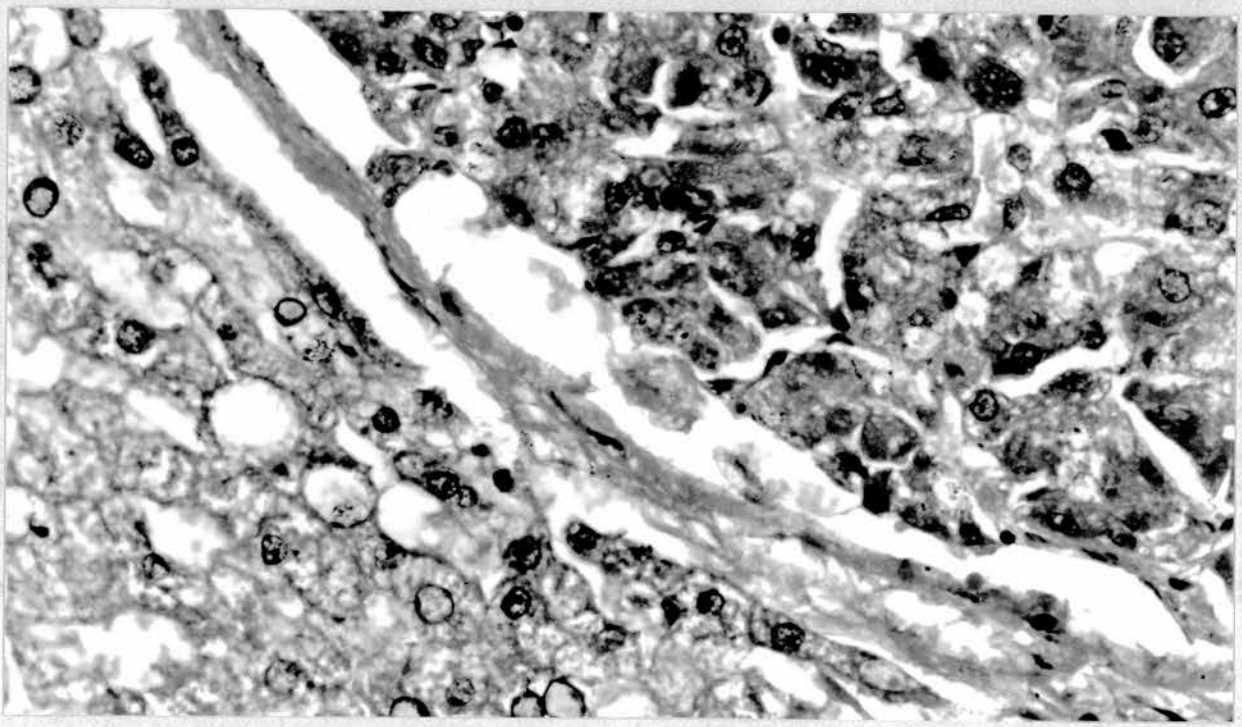
Abdominal ultrasound suggested that the whole right lobe of the liver was taken over by tumour and an echo-lucent area was seen in the left lobe. Serum alphafetoprotein was undetectable. Needle liver biopsy of the right lobe showed hepatocellular carcinoma (Table 3.2.a). Subsequent biopsies of liver less involved with tumour were examined - Table 3.2b. The glucose-6-phosphatase analysis suggested a diagnosis of type 1a glycogen storage disease.

Despite intensive supportive therapy, he died six days after admission.

At post-mortem, the liver filled most of the abdominal cavity and weighed 6.0kg - 10% of his body weight. A 140mm diameter tumour was visible in the right lobe and the rest of the liver had a variegated appearance with numerous yellow nodules scattered through the parenchyma. The liver histology is as in Table 3.2a and Figures 3.1 & 3.2. The lungs were very congested and contained many tumour emboli. The kidneys were enlarged and showed ischaemic damage with increased glycogen in the cytoplasm and nuclei of the tubular cells. The brain, intestine and pancreas were reported as normal. Skeletal muscle was macroscopically and microscopically normal. The post mortem report is copied in Appendix B.



a



b

Figure 3.1
Case 1. H&E stain of section of liver biopsy. (a) x50 (b) x500.A nodule of hepatocellular carcinoma is seen amongst liver parenchyma. Details of histology are given in the P.M report (page 211).

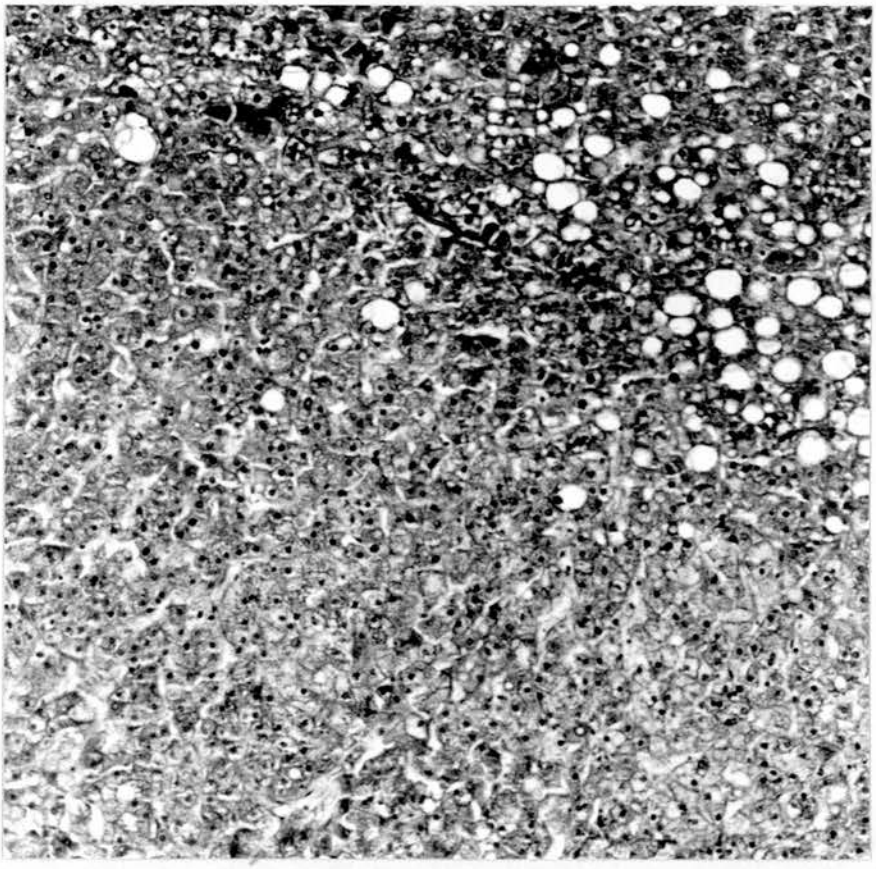


Figure 3.2

Case 1. H & E stain of liver x125 not involved with hepatocellular carcinoma obtained at post mortem.

Case 2

Female born 20.12.64. Presented in 1984 at the age of 19 with iron-deficiency anaemia. Extensive investigations were normal (including biopsies of rectal and duodenal mucosa and a small bowel barium meal) - the iron deficiency was attributed to menstrual loss and responded well to three months of iron replacement therapy and did not recur. She had mild post-exertional asthma requiring no regular therapy, had a nasal currettage at age 12 years for persistent rhinitis and benign familial tremor. The patient has one older sister who has had recurrent miscarriages. The patient's mother's obstetric history is unremarkable and there is no family history of neonatal deaths.

In 1986 at the age of 22, she presented with symptoms of loss of temper, light-headedness and hunger occurring between meals, especially if she ever missed a meal which she was doing at this time as she had left home to become a student of Town Planning. She had been born prematurely and her mother had had to feed her frequently up to the age of 15 years, after which time the patient's symptoms had worsened.

Examination apart from the tremor was unremarkable. There was no abdominal organomegaly. An oral glucose tolerance test was normal (Table 3.1). All routine serum biochemistry and haematology were normal. The prothrombin time was slightly prolonged (14.5 seconds, control 13 seconds) as was the kaolin cephalin clotting time (51 seconds, control 44 seconds). Fasting glucagon test showed a blunted blood glucose response - Table 3.3.

Liver biopsy was performed - the liver was hard at the time of biopsy. The histology of the liver is shown in Figure 3.3 and reported in Table 3.2a. Table 3.2b shows the biochemical analysis showing the patient to have a partial type 1a glycogen storage disorder. Her symptoms responded well to dietary advice.

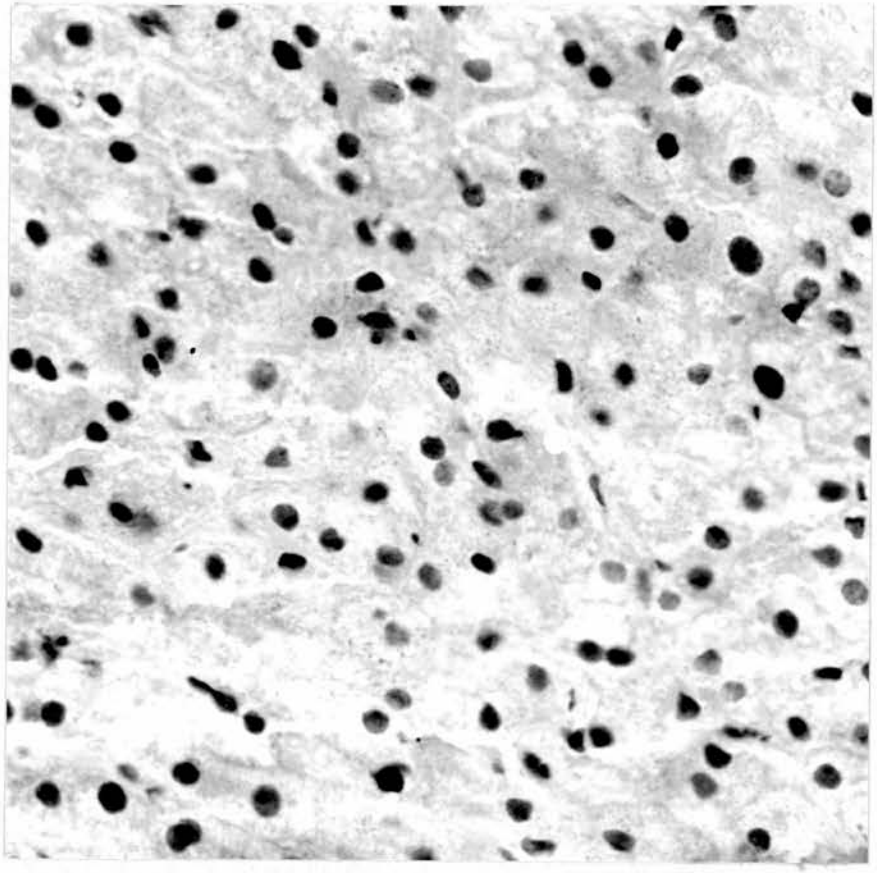


Figure 3.3

Case 2. H & E stain of a section of liver biopsy. x 660.

Small piece of liver only. Apparently normal architecture and no evidence of increased glycogen stores.

Case 3

Female born 11.05.34. As a child she had diphtheria and chorea secondary to scarlet fever. In 1955 when she was 21 years old she had her only pregnancy which was uncomplicated and delivered a normal female infant at term. In 1965 at the age of 31 she developed low back and knee pains. Orthopaedic examination and X-rays were normal and the symptoms responded well to strengthening exercises. In 1980 at the age of 46 fibrocystic breast disease was diagnosed.

In 1986 aged 51 she presented with a two month history of 9kg weight loss. There were no specific symptoms other than pain in the left medial epicondyle which was relieved by a local steroid injection. She had smoked on average 15 cigarettes per day for many years and took little alcohol at weekends only. Family history was unremarkable.

Clinical examination was normal - in particular there was no hepatomegaly and she was euthyroid. Glycosuria was not detected and random blood glucose was 4.4mmol/l. All other routine serum biochemical testing (including liver enzymes) was normal. ESR was 7mm in the first hour; full blood count and film were normal. Plain X-ray of the chest was normal. Upper abdominal ultrasound showed no focal liver defects and normal liver and kidneys.

At the next outpatient visit she had gained 2kg in weight and she gave a history of attacks of sudden tiredness occurring mid-afternoon which were worse if she was very active. Her lunch usually comprised a cup of coffee. These attacks had been occurring for many years and had worsened after the menopause. They were always relieved within ten minutes by eating a bar of chocolate.

A short synacthen test was normal. After 48 hours fasting and exercise, blood glucose was 3.1mmol/l and serum insulin $<$ 10mU/ml. Fasting glucagon test was performed and showed a blunted glucose response - Table 3.3.

Tests of in vitro blood clotting prior to liver biopsy showed prothrombin time equal to control, but a kaolin cephalin clotting time prolonged at 62 seconds (control 45

seconds). Further haematological investigation indicated a partial deficiency of Factor XI as the cause of the prolonged kaolin cephalin clotting time. Despite the lack of a clinical bleeding tendency, the liver biopsy was performed under cover of infused fresh frozen plasma without difficulty.

The histology of the liver biopsy is shown in Figure 3.4 and in Table 3.2a. Biochemical analysis (Table 3.2b) showed increased glycogen stores and diagnosis of a partial type 1a glycogen storage disease.

During these investigations she developed post-menopausal bleeding. Cervical smear was class 1 and subsequent cervical dilatation and uterine curettage were normal.

She was given dietary advice and oral hormone replacement therapy and the attacks lessened in severity and frequency. Seven months after diagnosis she defaulted from follow-up.

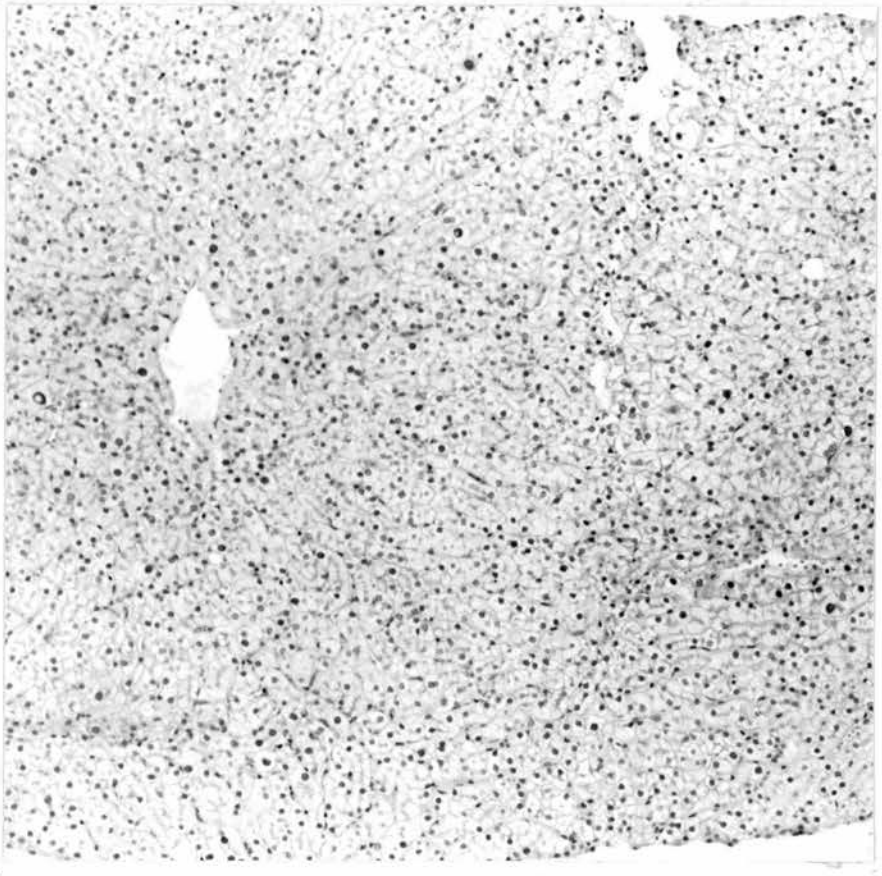


Figure 3.4

Case 3. H & E stain of sections of liver biopsy. x125. Normal liver.

Case 4

Female born 03.04.45. At the age of 25 in 1970 after a normal pregnancy a normal female infant was delivered spontaneously at term. This was her only pregnancy. At the age of 33 she had a course of radiotherapy for Stage IIb/III cervical carcinoma. Subsequent cervical smears up to 1989 were class 2 showing mild dyskaryosis only. Her other medical history was unremarkable. Unfortunately the patient is a poor historian with a dull affect and it was not possible to obtain detailed personal and family history.

In 1984, aged 39, she had a bout of non-specific abdominal pain - upper gastrointestinal endoscopy, barium follow-through and oral cholecystography were all normal. Her history included weight gain and flushing episodes worsened by exercise. Thyroid function was normal - total serum T4 (90 nmol/l) and TRH test (baseline 2.7mU/l, peak 5.3mU/l at 20 minutes).

In 1987 (age 42) she was seen with attacks of sweating followed by syncope. No temporal relationship to food was noted. An EEG was normal and no firm diagnosis was made. Her general practitioner prescribed a course of anti-depressant and sedative drugs.

In 1989 (age 44) she was seen with persisting, daily sweating attacks. The sweat started on her face and spread to the scalp. The episodes lasted longer in warm weather and were clearly induced by exercise. Occasionally the attacks progressed to palpitations and even loss of consciousness. There had been no witnessed convulsions. The episodes were not clearly terminated by ingestion of carbohydrate. There was no diarrhoea or wheeze during the flushing/sweating episodes although she was a heavy smoker and her chest was always a little wheezy.

Clinical examination showed her to be obese, have a dull affect and pigmentation of the skin under the eyes and of the buccal mucosa. There was mild proximal muscle weakness. A 1mg overnight dexamethasone suppression test excluded Cushing's syndrome (morning cortisol 43nmol/). Two outpatient urine collections showed high

excretion rates of 5HIAA (117 and 170μmol per day - normal < 40μmol/24 hours) but were noted to be of small volume. Six subsequent (in-patient) collections showed normal 24 hour urinary 5HIAA excretion. Thyroid function testing was again normal. Short synacthen test showed baseline serum cortisol 752nmol/l rising to 1242nmol/l. Serum oestradiol (< 70pmol/l), LH (47 U/l) and FSH (> 25 U/l) confirmed that she was post-menopausal. Serum liver enzymes were mildly raised - aspartate aminotransferase 87 U/l (normal < 30 U/l) and gamma glutamyl transferase 77 U/l (normal < 40 U/l). Serum levels of alkaline phosphatase, bilirubin and alpha alphafetoprotein were all normal. Full blood count was normal except for an MCV of 102 fl (no macrocytes were seen on the blood film). *In vitro* blood clotting studies were normal. Hepatitis B surface antigen, antinuclear factor, antimitochondrial antibodies and RA latex factor were not detected in the serum. An isotope liver scan showed a large liver with uniform uptake of isotope; upper abdominal ultrasound examination confirmed the homogenous texture of the liver parenchyma.

The fasting glucagon test showed a blunted glucose response (Table 3.3). Figures 3.5 and 3.6 and Tables 3.2a and 3.2b contain the results of the liver biopsy. Biochemical analysis (of a small piece of liver) showed no glucose-6-phosphatase activity and absent protein by immunoblotting suggesting type 1a glycogen storage disease.

Treatment with diet and oestrogen hormone replacement therapy have relieved most of her symptoms.

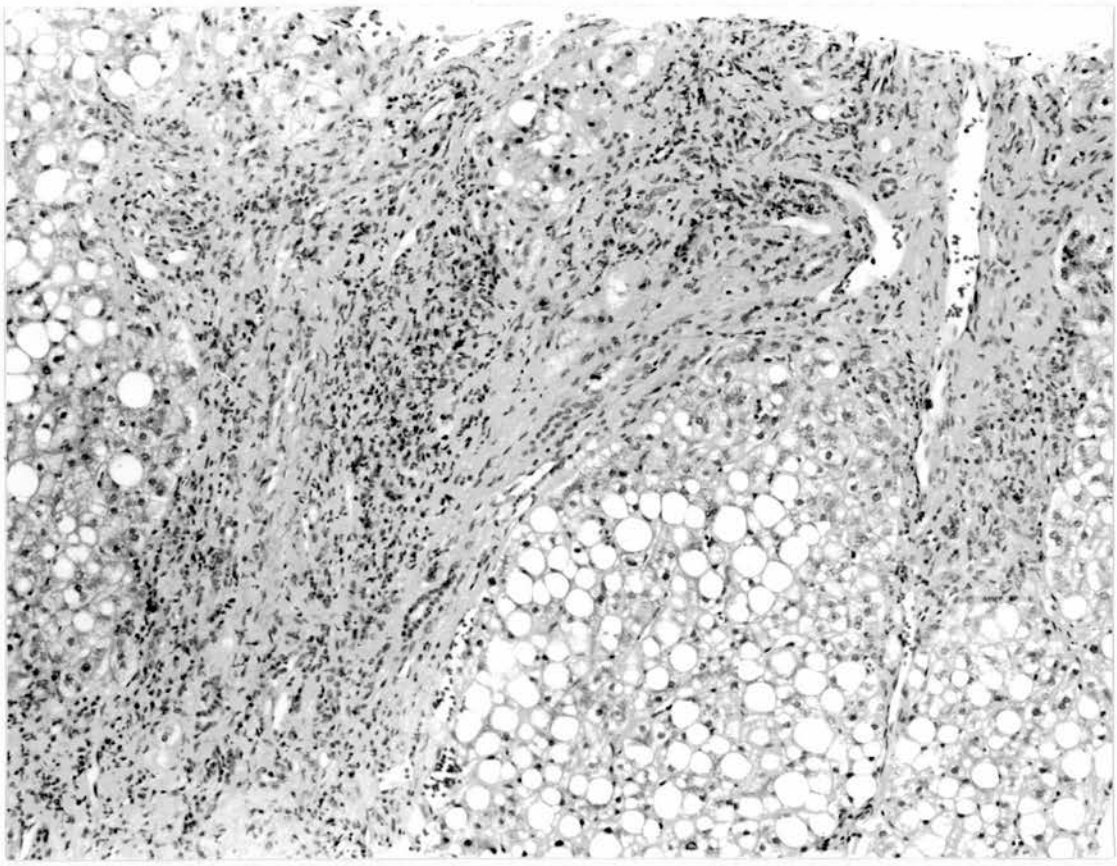


Figure 3.5

Case 4. H & E stain of sections of liver biopsy. x125. There is an excess of fibrous tissue extending from the portal tracts which disturbs the hepatic architecture., suggestive of micronodular cirrhosis (the specimen is too small to show any nodular collections). Within the fibrous tissue and extending into the parenchyma there is a moderate chronic active inflammatory infiltrate. No Mallory's hyaline is seen. The features are of a moderately severe steatohepatitis with possible cirrhosis.

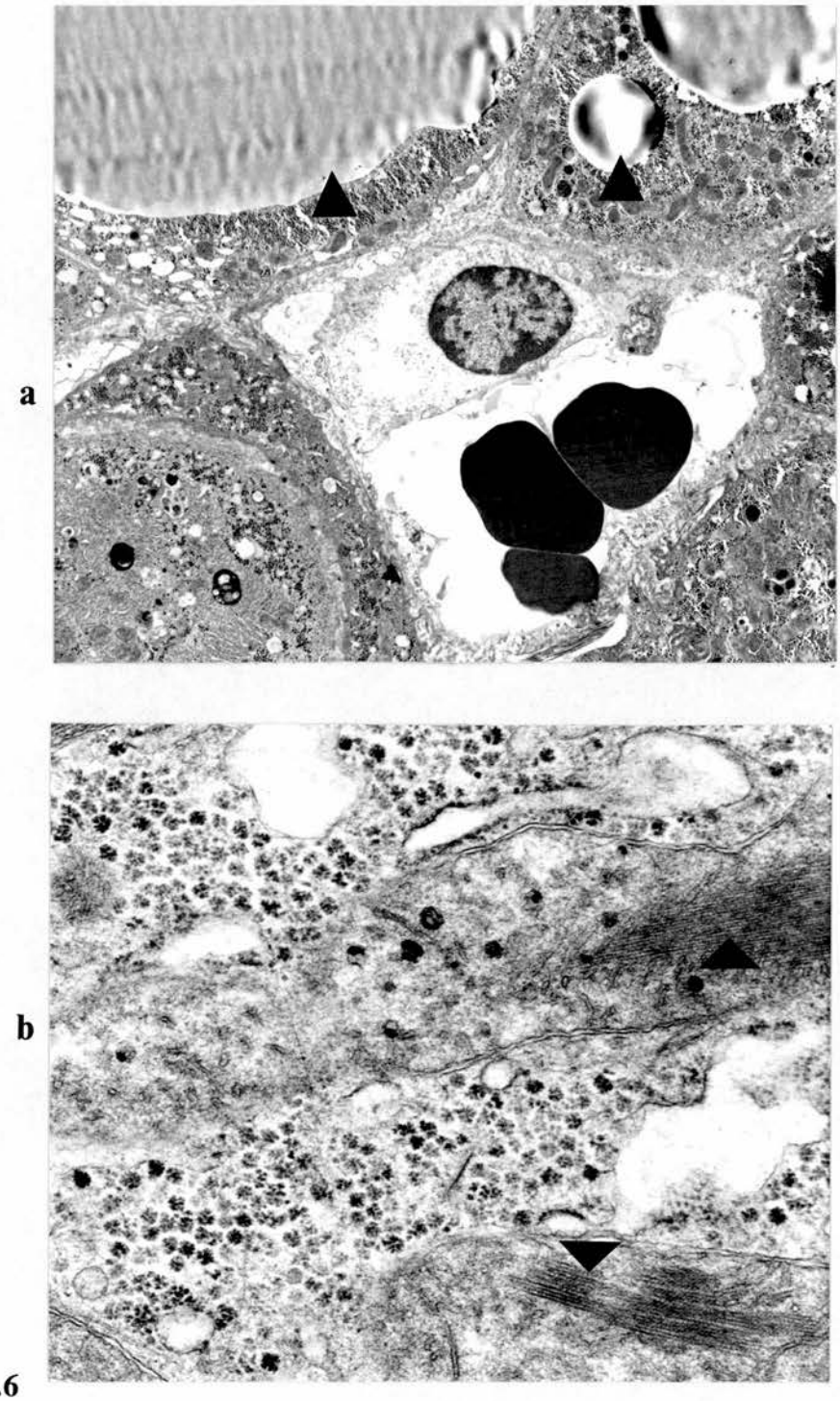


Figure 3.6

Case 4. (a) and (b) Electron micrographs from liver biopsy.

(a) x3,200. Generalised fatty change (lipid droplets arrowed). Generalised increase in glycogen (seen as black stippling) throughout hepatocytes.

(b) x32,000. Glycogen stores seen more clearly (black rosette pattern) and mitochondrial paracystalline arrays are seen (arrowed).

Case 5

Female born 03.03.35. In 1976 at the age of 41 years she was seen at another hospital complaining of episodic weakness mainly after exercise which improved within ten minutes of ingesting glucose tablets. Clinical examination was unremarkable. After a 60 hour fast blood glucose was 2.4mmol/l (serum insulin was not measured) and the patient was asymptomatic during the fast. A 75g oral glucose tolerance test was performed (Table 3.1). An abnormality of hepatic glucose output was considered and a fasting glucagon test was performed (Table 3.3); the absent blood glucose response was not commented upon. Advice was given to cover exercise with glucose and no further investigations performed.

In 1986 aged 51 years she presented first to this hospital giving a 20 year history of episodic weakness, especially of the lower legs, associated with exercise and associated with incoordination, light headedness and tremulousness. These episodes were often, but not invariably associated with exercise and were always relieved within ten minutes by ingestion of glucose tablets. The menarche is not known, but her periods were always regular. She had had one pregnancy which produced a normal female infant (who went on to become a medical practitioner). Her last menstrual period had been in 1985 and her symptoms had worsened since then. She owned a part share in a book shop which she helped run. Her only past history was of a myomectomy at the age of 39 years. Physical examination was normal.

Fasting glucagon test was repeated (see Table 3.3) and this time a (subnormal) rise in blood glucose was measured. A short synacthen test was normal (rise in serum cortisol 294nmol/l). Haematological, clotting and serum biochemical investigations were normal. Fasting total cholesterol was 6.8mmol/l and triglycerides 0.91mmol/l. Liver biopsy was performed and the biochemical analyses are shown in Tables 3.2a and b. The biochemical analysis shows her to have a partial type 1a glycogen storage disease. Photographs of histology and electron microscopy of the biopsy are shown in Figures 3.7 and 3.8.

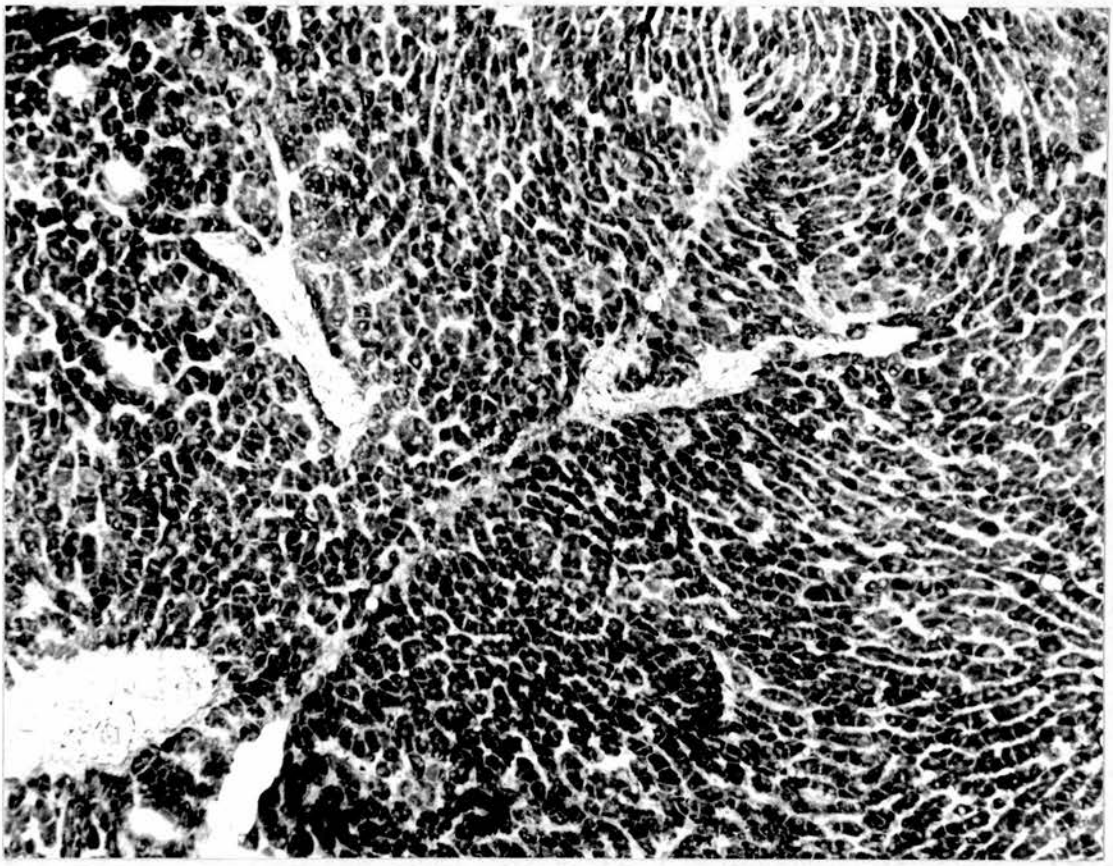
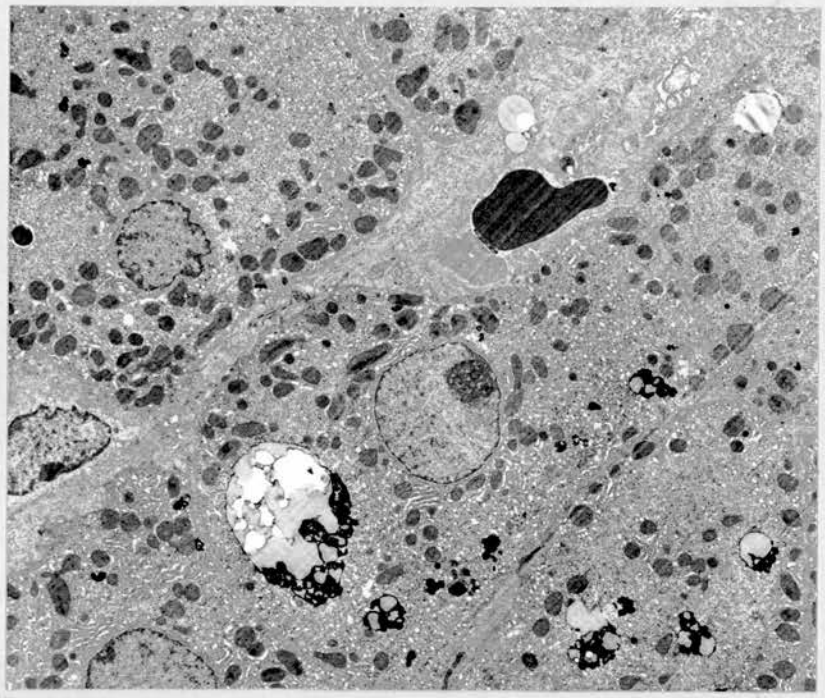


Figure 3.7

Case 5. Section of liver from biopsy stained for glycogen. x125. Excess of glycogen uniformly distributed across all zones. (H & E showed some increased lipofuscin in the perivenular hepatocytes, but was otherwise normal).

a



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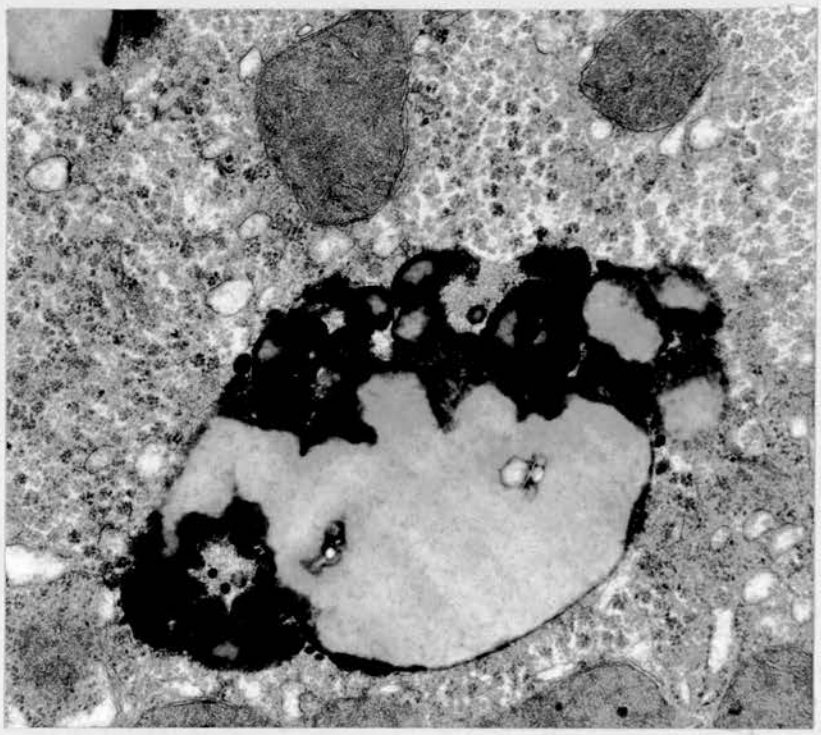


Figure 3.8
Case 5. Electron micrographs of liver biopsy. (a) x2,600 and (b) x26,000. There is an excess of glycogen with the organelles distributed uniformly (a). The sinusoidal borders are normal as are the bile cannaliculi although these contain particulate material. Some mitochondria are enlarged and contain paracrystalline arrays (not shown). There is some dilatation of the rough and smooth endoplasmic reticulum. The Golgi apparatus and nuclei show no significant abnormality. There are a few fat droplets, some of which are incorporated in the residual bodies (b). The littoral cells are within normal limits.

Her symptoms improved with dietary advice.

Her mother and sister had similar symptoms; her sister subsequently had a normal glucagon test; as her mother was over 80 years of age it was not felt appropriate to investigate her.

Twelve months later she presented with flushing unrelated to exercise or meals. This was successfully treated with hormone replacement therapy (Loestrin 20, later changed to Prempak). A short time later, after a viral illness, she developed marked tiredness of her lower limbs. There was no objective muscle weakness or clinical neurological deficit. Electromyographical studies and muscle biopsy of affected muscles were normal. Despite lack of serum viral antibodies, this illness was attributed to post viral fatigue syndrome and the symptoms improved over the subsequent three months.

More recently she has developed a thyroid nodule which has been aspirated and shown to be cystic with benign histology. She has remained clinically and biochemically euthyroid.

Case 6

Male born 05.05.33. In 1986 at the age of 53 he presented with polydipsia, polyuria and nocturia, but no weight loss. He was a heavy smoker (40 cigarettes per day for most of his adult life) and had drunk at least 30 units of alcohol per week until 1984 since when he stopped after his General Practitioner found mildly elevated serum liver enzyme levels.

He worked as a labourer with the council Parks Department and was married with a son born in 1964. On examination he was overweight (105.5kg, 1.79m tall), hypertensive (186/100mm Hg, phase V) and had 2% glycosuria. He had palmar erythema and several spider naevi. The liver was easily palpable, but not clinically enlarged.

Random blood glucose was 9.9mmol/l; an oral glucose tolerance test showed a diabetic pattern (see Table 3.1). Random serum cholesterol was 5.93mmol/l. Serum biochemistry was normal except for alkaline phosphatase 175 U/l (normal < 120 U/l). Haematology and blood film were normal. Hepatitis B surface antigen, antinuclear factor, antimitochondrial and anti smooth muscle antibodies were not detected in serum. Isotope liver scan showed a moderately enlarged liver with uniform uptake. Chest X-ray was normal.

Liver biopsy was performed in view of the large liver. Only a very small piece of tissue was received for biochemical analysis which showed normal disrupted glucose-6-phosphatase activity but the activity in intact structures was too low to be properly assessed (Table 3.2b). The glycogen content of the fragment was high. As this abnormality was unexpected but potentially clinically important a wedge liver biopsy was performed at laparotomy. The glucose-6-phosphatase analysis (Table 3.2b) and pyrophosphatase assay data (Table 3.2c) are characteristic of type 1c glycogen storage disease.

A fasting glucagon test was subsequently performed (Table 3.3) and was normal. Follow-up random blood glucose levels at diabetic clinics were normal. At no time, on a diabetic weight-reducing diet did he develop symptoms of hypoglycaemia.

The possibility of a Fanconi-type syndrome of proximal renal tubular damage was considered if the renal tubular cell phosphate transporters were abnormal too. However he had a normal renal phosphate threshold at 1.16mmol/l (normal 0.8 - 1.35mmol/l) with <0.1g/l proteinuria and no amino aciduria.

In 1989 he developed a painful lytic lesion in the left acromion process - biopsy revealed anaplastic small cell carcinoma, probably metaplastic from a bronchogenic primary. Chest X-ray showed slight mediastinal widening but bronchoscopy was normal. Isotope bone scan revealed widespread bony metastases. Palliative radiotherapy to the painful bony lesions and pulsed chemotherapy were started, but he died in July 1989. Permission for post-mortem examination was not given.

Case 7

Female born 24.04.69. Presented as a University student in 1989 at the age of 20 years with two or three episodes per day of blurring of vision, shakiness, illegible handwriting and slurred speech. These episodes occurred usually before meals and were relieved by eating. The symptoms had worsened since leaving home (and eating less regularly) to come to University. Her general practitioner had given her blood glucose testing sticks and with these she had demonstrated a blood glucose less than 2mmol/l during one of these attacks. She had been vegetarian for 4 years, did not smoke and took no alcohol. Her past medical history was unremarkable. She had been born three weeks premature, but had developed normally. Her mother's other obstetric history was unremarkable. There are no siblings. Her menses were regular with a 21 day cycle. Later she complained of painful, heavy periods. Clinical examination showed her to be overweight but was otherwise unremarkable. During a 48 hour fast she often complained of symptoms of light headedness and blurring of vision but at no time was her blood glucose less than 3.5mmol/l. She was exercised at the end of 48 hours fasting and subsequent blood glucose was 3.2mmol/l, serum insulin 10mU/l. Blood glucose response to glucagon in the fasting state was impaired (Table 3.3). Oral glucose tolerance test showed impaired glucose tolerance (Table 3.1). Fasting lipids were normal (total cholesterol 5.38mmol/l, triglyceride 1.3mmol/l). Haematological testing (including in vitro clotting studies) was normal.

The histology of the liver biopsy is shown in Figure 3.9.

The biochemical results of the liver biopsy are presented in Tables 3.2a,b and c. The kinetic data indicate that all components of the glucose-6-phosphatase system are present in the correct proportions, but the activity of each part and of the system as a whole is low. The glucose-6-phosphatase data is very unusual and had never been seen before in an adult, although it is a pattern recognised in some premature infants.

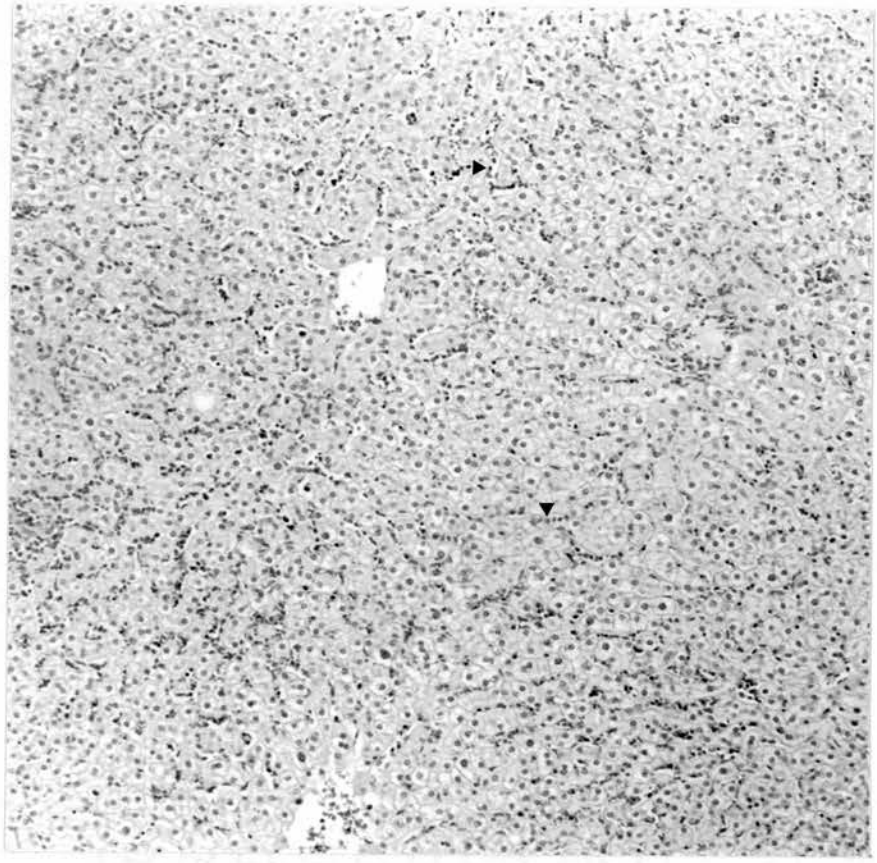


Figure 3.9

Case 7. H & E stain of section of liver biopsy. x125. Portal tracts expanded by infiltrate of lymphocytes (some with a centrocytic appearance), PMNs and eosinophils. In the sinusoids are some collections of lymphocytes arranged indian file (arrowed).

Electron microscopy (blocks no longer available) shows some fatty droplets in hepatocytes and lipid-containing residual bodies. Mitochondria contain paracrystalline arrays. In some cells the organelles are pushed to the periphery (by glycogen). Some bile cannaliculi contain particulate debris as does a bile duct.

The liver histology was suggestive of a viral illness with lymphocytic infiltration but there was no serological evidence of an acute viral illness - hepatitis A and B serology were negative, but there was circulating IgG to the Epstein-Barr virus. The patient always appeared very anxious, but eventually responded very well to dietary manipulation.

At the age of 21 she presented with a 3 month history of colicky abdominal pain and frequent looser-than-usual bowel motions. Examination and investigations suggested a diagnosis of irritable bowel syndrome. These symptoms resolved spontaneously some months later.

Case 8

Male born 1940. Led a very active working (farmer) and sporting life, playing rugby and cricket into his thirties. He presented aged 42 years with symptoms suggestive of reactive hypoglycaemia, worst between 1000 and 1030 h after an early rise (0500 h) and heavy breakfast (0900 h). These symptoms were precipitated by physical activity and were relieved by the ingestion of food. There was no past history or family history of note. He did not smoke and alcohol consumption was less than 1 unit per day. Physical examination was unremarkable.

A prolonged fast showed at 48 hours an insulin:glucose ratio of 0.12mU/mmol (normal $<$ 0.3mU/mmol). A prolonged 75 g oral glucose tolerance test (OGTT) (Figure 3.10) produced symptomatic hypoglycaemia. A diagnosis of reactive hypoglycaemia was made and dietary advice given.

At age 47 years, he was re-referred with worsening hypoglycaemic symptoms in the same pattern as before. Other history and clinical examination were unremarkable. OGTT demonstrated a large rise in total serum insulin (peak 720mU/l) with a small rise in plasma glucose (Figure 3.11i).

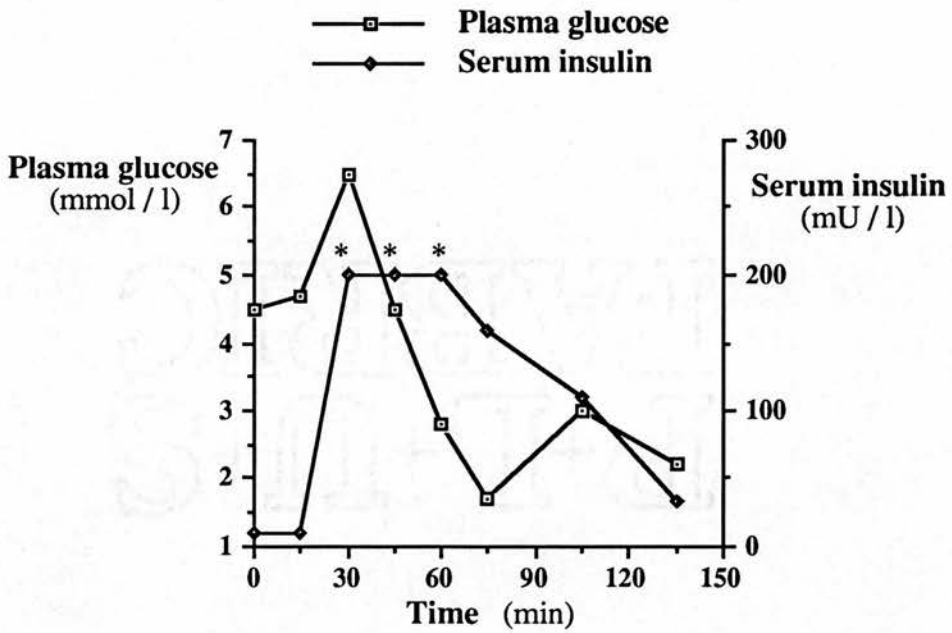


Figure 3.10

Oral glucose tolerance test performed on case 8 then aged 42 years. (* - further dilutions of serum for insulin assays were not performed).

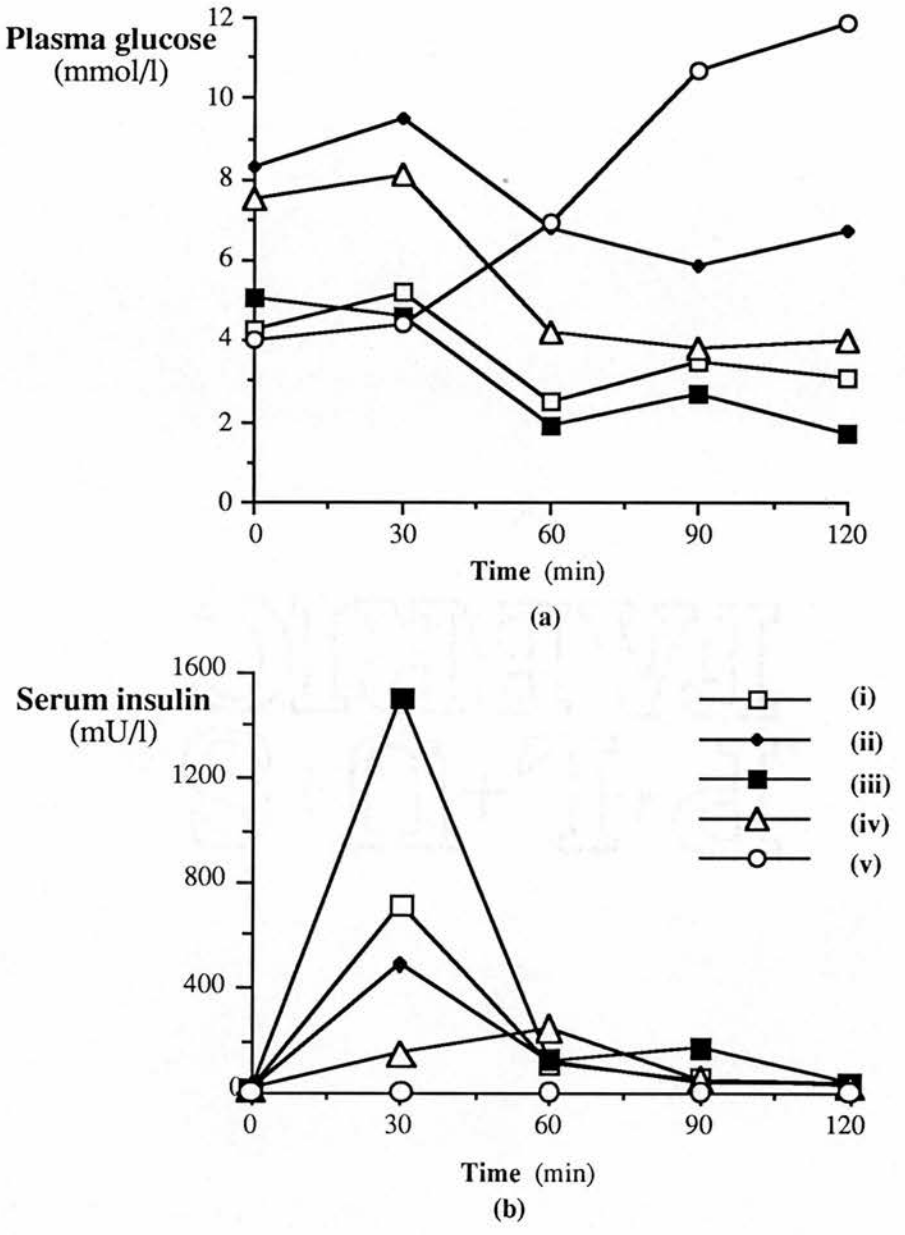


Figure 3.11

Results of a series of oral glucose tolerance tests performed on case 8 now aged 47 years. Plate (a) shows the blood glucose levels and plate (b) the serum insulin levels.

(i) At presentation, pretreatment; (ii) taking 900mg diazoxide per day; (iii) taking 100mg diazoxide x2 per day; (iv) taking 200mg diazoxide x2 per day; and (v) after a single dose of octreotide 100μg still taking 200mg diazoxide twice per day.

An intravenous glucose tolerance test was performed. This produced severe, profound hypoglycaemia after 90 minutes, following hyperinsulinaemia, and a virtually absent glucagon counter-response (Table 3.5). Other counter-regulatory hormone responses to hypoglycaemia were normal (including adrenaline and noradrenaline, peak levels of both 3nmol/l at 120 mins). The test had to aborted to revive the patient from his profound hypoglycaemia. He became drowsy and had to be given small IV boluses of glucose and IV hydrocortisone and adrenaline until such time as he was able to eat.

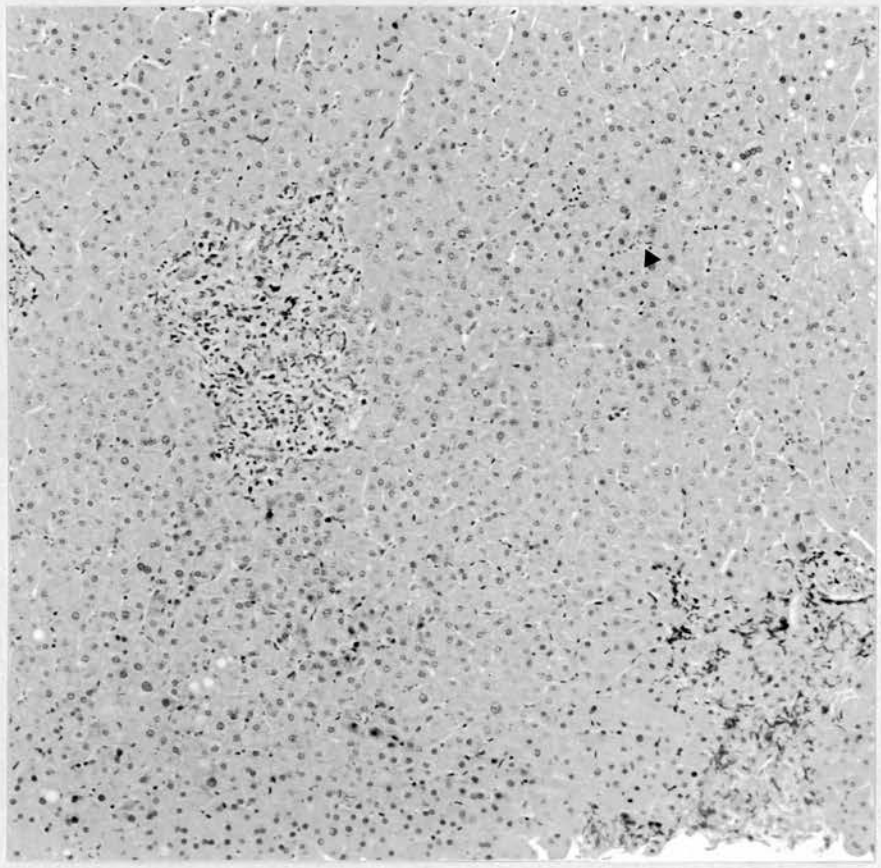
A short synacthen test was normal (baseline serum cortisol 355nmol/l, peak 1000nmol/l).

A fasting glucagon test showed a blunted rise in blood glucose levels followed by hypoglycaemia (Table 3.3). This test suggested abnormal hepatic glucose production so a needle liver biopsy was performed after an overnight fast. Liver histology was normal (except for lymphocytic infiltration of the liver initially taken to represent a lymphoma - see Figure 3.12 and Discussion below) as was the measured glycogen content of 27.2mg glycogen/g wet liver weight (normal <40mg/g). Hepatic glucose-6-phosphatase kinetics were measured on a microsomal subfraction (Table 3.2b).

The high Km in intact microsomes, restored to normal by pre-incubating the microsomes with 1g/l bovine serum albumin (BSA), implies that there is a reversible inhibitor of T1 binding to the transport protein in this patient's liver preparation. BSA is a non-specific binder of many ions and peptides and thus the nature of this inhibitor is not clear. The activity of the catalytic subunit (as indicated by the disrupted assays) was normal.

CT scan of the pancreas before and during rapid infusion of contrast medium did not reveal any abnormality.

a



b

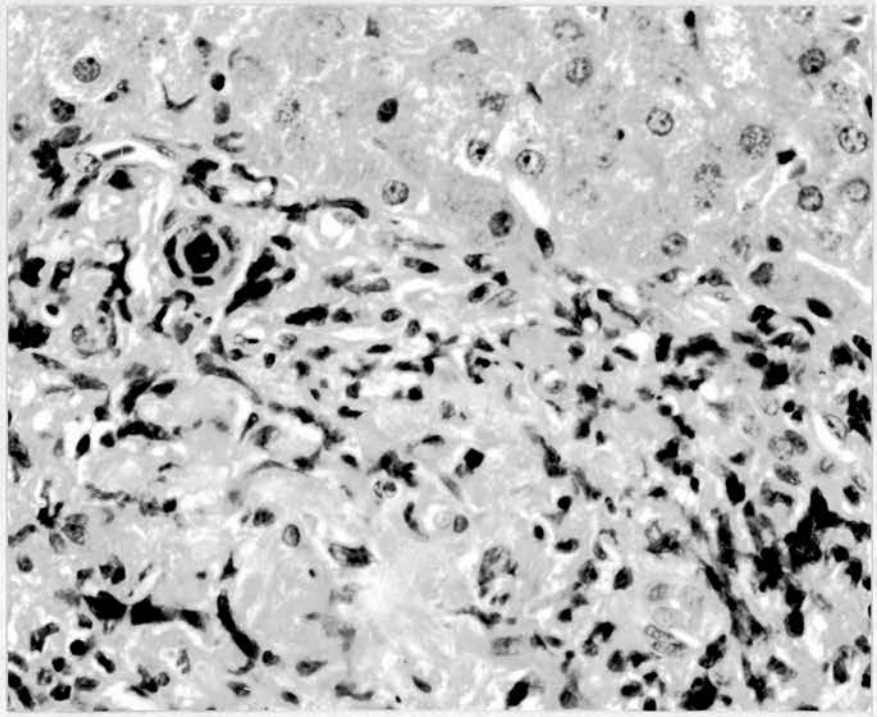


Figure 3.12
Case 8. H & E stain of sections of liver biopsy. (a) x125, (b) x660. (a) Sections show extensive infiltration of the portal tracts by a mixed lymphocytic histiocytic lymphoma. Portal tract magnified in plate (b) to show close up of infiltrate. Sinusoids show some lymphocytes arranged indian file (arrowed).

In view of the marked hyperinsulinaemic response to glucose, treatment with oral diazoxide was commenced at 900mg/day. This gave good relief of hypoglycaemic symptoms within 14 days.

Repeat OGTT performed after 14 days of 900mg/day diazoxide showed raised fasting and peak plasma glucose levels but a reduced rise in serum insulin concentration (peak 485mU/l) (Figure 3.11ii).

The dose of diazoxide was reduced to 50 mg twice a day to alleviate the side effects of fluid retention and hypertension and then gradually increased to 200 mg twice a day with clinical improvement. OGTT was repeated 8 weeks after starting 400 mg/day diazoxide and although fasting and peak plasma glucose levels were elevated compared with prior to therapy the peak rise in insulin was reduced (Figure 3.11iii).

While on 400 mg/day diazoxide the patient was given a single dose of 100 μg octreotide (Sandoz Pharmaceuticals, Camberley, UK) by IM injection and the OGTT repeated. The insulin response to oral glucose was abolished (Figure 3.11iv), but the patient suffered abdominal discomfort of such severity he was reluctant to use the drug regularly.

Five years later the patient remains symptomatically well on diazoxide 200 mg twice a day and has octreotide at home for emergency use (as yet unused).

Table 3.1

Results of oral glucose tolerance tests. 75g glucose given orally after > 10 hour fast

(Results are venous plasma glucose levels in mmol/l)

		Time (mins)					
Patient No.	Fasting	30	60	90	120	150	180
1*	3.0	6.0	6.9	5.0	3.7	NS	NS
2	4.5	7.4	9.4	9.4	8.5	NS	5.6
3	NP**						
4	NP**						
5	4.1	5.0	3.9	3.6	3.5	3.5	4.2
6	5.6	11.6	13.0	9.8	6.8	NS	NS
7	4.2	10.1	12.2	12.2	9.3	NS	NS
8	4.0	2.0	<0.5	2.6	2.7	NS	NS

* GTT performed in 1966. Amount of glucose given not known

**NP = GTT not performed

NS = No sample

Table 3.2a

Histological examination of liver biopsies from patients

Patient	Comment	Glycogen content seen by PAS
1	Hepatocellular carcinoma. Massive glycogen stores in remaining liver	3+
2	Normal liver	+
3	Normal liver	+
4	Marked fatty change, moderate portal fibrosis. Moderate chronic active infiltrate	+
5	Increased perivenular lipofuscin	3+
6	Mild fatty change. Increase in portal tract lymphocytes	+
7	Excess lymphocytes in portal tracts	3+
8	Mixed lymphocytic histiocytic infiltration	3+

+ represents normal glycogen stores 3+ represents markedly increased glycogen stores

Table 3.2b

Glucose-6-phosphatase activity in hepatic biopsy specimens from cases presented

Patient	Intact microsomes		Disrupted microsomes		Glycogen content
	Vmax (mmoles/min/mg)	Km (mM)	Vmax (mmoles/min/mg)	Km (mM)	(mg glycogen/g wet weight liver)
1	0	ND	0	ND	134
2	0.01	1.7	0.02	1.1	70
3	0.01	3.3	0.04	1.0	82
4	0	ND	0	ND	166
5	0.03	3.3	0.08	1.3	96
6	0.09	6.9	0.53	1.1	80
7	0.01	2.2	0.06	1.0	54
8	0.03	16.2	0.2	0.9	28
	0.15*	2.2	0.2	0.9	
Control values(n=60)	0.23±0.02	2.6±0.2	0.35±0.03	0.8±0.08	<40

Control values are mean ± SEM. Values indicate that there is a skewed distribution of data .

ND = Not determinable

* = Microsomes preincubated with 1% BSA

Table 3.2c

Pyrophosphatase activity in microsomes from hepatic biopsy specimens from cases presented

Patient	Intact microsomes		Disrupted microsomes	
	Vmax (mmoles/min/mg)	Km (mM)	Vmax (mmoles/min/mg)	Km (mM)
1	0	ND	0	ND
5	0.01	3.2	0.04	2.3
6	0	ND	0	ND
7	0.004	1.7	0.07	1.0
Control values (n = 25)	0.08±0.001	2.5±0.32	0.24±0.02	0.7±0.1

Control values are mean ± SEM. Values indicate that there is a skewed distribution of data .

ND = not detected

Table 3.3

Results of glucagon tests in cases presented. 1mg glucagon given by IM injection after overnight fast.

Patient	Venous plasma glucose (mmol/l) Basal	Peak	Rise in blood glucose	Time to peak (mins)
2	4.4	7.4	3.0	60
3	5.2	7.4	2.2	30
4	5.6	9.1	3.5	60
5a*	4.6	4.6	0	0
5b**	5.3	7.4	2.1	30
6	5.9	11.1	5.2	60
7	4.6	6.1	1.5	30
8	4.6	6.6	2.0	30

NP - glucagon test not performed

* a) - Performed 1976

** b) - Performed 1986

Table 3.4

Results of an intravenous GTT (0.5 g/kg body weight) in case 8

Time (min)	Plasma glucose (mmol/l)	Serum insulin (mU/l)	Serum growth hormone (mU/l)	Serum cortisol (nmol/l)	Serum glucagon (pmol/l)
0	4.1	20	3.6	178	5
15	14.3	408	1.1	507	5
30	9.5	400	1.0	554	<5
45	6.6	479	0.9	537	5
60	3.5	1520	0.8	454	5
90	1.2	268	0.8	360	5
120	1.1	96	> 62.5	885	11

Discussion

The existence of these cases is not surprising - all metabolic defects involving enzyme abnormalities have a spectrum of severity of presentations. The ability to diagnose such cases is new: using refined assay techniques and the confidence of a large pool of "normal" data (Dr. Ann Burchell's laboratory has assayed over 600 normal and abnormal adult and children liver samples with the sensitive assays described above). The literature on type 1 GSD contains reports of patients with no measurable glucose-6-phosphatase activity in liver biopsies, but varying severity of symptoms and measurable hepatic glucose production using stable isotope flux measurements (Farber *et al* 1976, Kalhan *et al* 1982, Tsalikian *et al* 1984 and Collins *et al* 1990). Other patients diagnosed as having "pseudotype 1 GSD or type 1 *bis*glycogenoses" (Nordlie & Sukalski 1986): patients with symptoms of type 1 GSD and "normal" assayed glucose-6-phosphatase activity (Rosenfeld *et al* 1978) and the heterogeneity of type 1b GSD patients based upon their clinical features (Tada *et al* 1983) and how they handle $^{3}H/^{14}C$ labelled glucose infusions (Schaub & Heyne 1983) may all represent partial deficiency states of proteins of the glucose-6-phosphatase system. The assay data on the liver samples in these reports have not been performed with sufficient substrates, have not been sensitive enough nor interpreted in the light of sufficient normal data to allow such a refined diagnosis to be made.

The clinical features of case 1 are very unusual. He was described as mentally backward - it is possible that this was due to recurrent hypoglycaemia and it is disappointing in retrospect that his brain was not more carefully examined at post mortem. All the other cases above appeared to have achieved full mental, physical and sexual maturation. The woman case 4 was not a good verbal communicator and had a very flat affect which again may be secondary to chronic hypoglycaemic brain damage. Case 1 was short in stature and there were repeated suggestions of poor sexual maturation which again may be related to hypoglycaemia during

development. He had long-standing hepatomegaly as would be expected with type 1 GSD - only 1 other of the patients (case 6) had a palpable liver (which may have been caused by the fatty liver of diabetes or his heavy alcohol intake), although case 4 had a large liver as judged by isotope scanning. Case 1 alone had a long-standing arthropathy which was probably gout which is a recognised long-term effect of type 1 GSD, and is the only similarity of case 1 with another case of type 1 GSD diagnosed in an adult woman with chronic tophaceous gout (Stamm & Webb 1975). This woman was a school-teacher who had had three children: in this respect she is more like cases 2-5 above.

Cases 1 to 5 above represent different point mutations or deletions in or differing expression of the gene(s) encoding the glucose-6-phosphatase catalytic subunit protein. Only cases 1 and 4 did not have a microsomal protein which cross-reacted to the polyclonal antibody against the rat hepatic microsomal catalytic subunit (see Pears *et al* 1992).

Case 1 above showed complete absence of cross-reactivity with antibody raised to the glucose-6-phosphatase catalytic subunit on immunoblot analysis, indicating complete absence of that protein. Immunostaining is a very sensitive technique and would have identified even very small amounts of the catalytic subunit in the liver of case 1. There is good cross-reactivity of this antibody between the rat hepatic microsomal glucose-6-phosphatase catalytic subunit and the catalytic subunit in normal human liver preparations (Burchell *et al* 1988b). Case 1 must have had some hepatic glucose production to have lived as long as he did: the only other way that the liver can produce glucose is via substrate cycles and debranching enzyme activity; no other phosphatase in hepatocytes has been identified which can hydrolyse glucose-6-phosphate.

Case 4 is similar to case 1 in that the catalytic subunit protein was not identified by immunoblotting. The reason why the woman in Case 4 did not have hyperuricaemia

or any of the other recognised metabolic features of type 1 GSD (see Hers *et al* 1989 & Moses 1990 for recent review) is not clear.

Cases 2-8 inclusive had, by immunoblot analysis, normal levels of normal molecular weight glucose-6-phosphatase catalytic subunit protein (Pears *et al* 1992). The defect in these cases was therefore functional. The molecular level at which the defect occurred is unclear; the chromosome(s) which contain the genes for the proteins of the glucose-6-phosphatase system have not yet been identified and cDNA probes do not exist for the glucose-6-phosphatase catalytic subunit and the sequence of the human catalytic subunit protein is unknown.

None of the subjects other than case 1 had clinical evidence of hyperlipidaemia and in retrospect it would have been interesting to classify in detail the lipid profiles in these subjects. The evidence from children with type 1a GSD is that although the lipid abnormalities (especially the hypertriglyceridaemia) improve to some extent with dietary manipulation aimed at reducing hypoglycaemia, later there will be an "escape" resulting in a marked rise in plasma triglycerides and cholesterol (Fernandes *et al* 1979). Therefore it may still be possible (and important) to demonstrate lipid abnormalities in the plasma of these patients some years after commencing dietary therapy.

Another abnormality commonly noted in severe type 1 GSD patients is a bleeding tendency, manifest often as epistaxes and excessive haemorrhage after surgery (Moses 1990). Type 1 GSD patients can be shown to have prolonged bleeding times and impaired platelet function (Gilchrist *et al* 1968, Czapek *et al* 1973, Green & Ratnoff 1974, Corby *et al* 1974 and Ambruso *et al* 1985). This defect is probably acquired as it improves markedly with dietary treatment (Czapek *et al* 1973 and Moses 1990). Only case 1 above had clinical episodes of bleeding, but on 2 occasions detailed coagulation studies were normal. Case 3 was found to have a deficiency of factor XI but only on the routine coagulation tests performed before a liver biopsy. She gave no clinical history of bleeding tendency. The likelihood of

the same individual having two protein defects are very small, but it is difficult to perceive a link between glucose-6-phosphatase and factor XI levels. The only abnormality of coagulation factors described before in type 1 GSD is an elevation of factor VIII levels in a single patient (Green & Ratnoff 1974).

The development of hepatomas (Poe & Snover 1988) and hepatocellular carcinoma first described in 1955 (Mason & Anderson, Limmer *et al* 1988 & Moses 1990) are amongst the most sinister complications of type 1 GSD. Screening using circulating αalphafetoprotein levels is unreliable (Limmer *et al* 1988 and case 1 above) so patients should have regular ultrasound or CT visualisation of their liver. Any increase in risk treating our patients with ovarian hormone replacement therapy on the development of hepatic adenomata must be awaited (oral contraceptive usage is associated with increased risk of developing hepatic adenomata). The likelihood is that the adenomata in type 1 GSD (which are usually multifocal) undergo malignant transformation to develop hepatocellular carcinoma. There has been a single report of decrease in size of hepatic adenomata in type 1 GSD with intensive dietary therapy (Parker *et al* 1981) but not of a reduced likelihood of malignant transformation. As yet none of the cases other than case 1 above have been shown to have liver tumours. The patients above are being followed wherever possible by Dr. Roland Jung.

It is possible that other tumours could be more common in type 1 GSD - two of the patients described here had carcinomas (cervix case 4 and bronchus case 6). However, these are relatively common cancers and their development in these patients may be co-incidental. There is no evidence of a defect in immuno-surveillance in type 1a GSD (unlike in type 1b where there is abnormal neutrophil function). However it is interesting to note the lymphocytic infiltration seen in three of the cases' liver biopsies (numbers 6,7 & 8) and to wonder whether this reflects abnormal lymphocyte function. The initial liver biopsy appearances of cases 7 and 8 was taken to represent lymphomatous infiltrate, but this opinion was later revised.

Only one case of a haematological malignancy has been described in type 1 GSD and that was a case of acute myelogenous leukaemia in a patient with type 1b GSD (Simmons *et al* 1984). Dr David Hopwood in the Department of Pathology in Dundee has looked retrospectively at liver biopsies of patients with lymphoma and was unable to demonstrate abnormally high glycogen deposits (personal communication). Note however that the biochemical analysis of the glycogen content of the liver in case 8 showed normal glycogen stores, and the histological estimate of glycogen content can be misleading.

Case 4 showed a chronic active infiltrate involving the fibrous septae and parenchyma in the liver. The pathogenesis and significance of this is unclear.

Renal impairment (another sinister long-term complication of type 1 GSD [Baker *et al* 1989 and Chen *et al* 1988 & 1990]) has not been seen in any of our patients other than case 1 at the time of his terminal illness. However the development of renal failure in type 1 GSD patients can be very rapid and often follows a period of hyperfiltration similar to that seen in incipient diabetic renal failure (Baker *et al* 1989). The development of proteinuria ($\pm$ hypertension) and renal impairment is associated with focal segmental glomerulosclerosis on renal biopsy and seems to begin in the teenage years in patients with severe type 1 GSD and to have been preceded by poor treatment or frequent feeds only - the early evidence is that the use of corn starch or nocturnal nasogastric feeding prevents (or delays) the development of proteinuria (Chen *et al* 1988). There is also evidence of proximal tubular disease (amino aciduria, increased $ß_2$microglobulinuria, and decreased phosphate reabsorption) in some type 1a GSD patients (Chen *et al* 1990). This was not, however, evident in case 6 above where it was actively sought - as his glucose-6-phosphatase abnormality was a defective hepatic microsomal phosphate transporter, we wondered whether the phosphate transport system in his renal tubules may also have been affected. The presence or absence of T2 in kidney microsomes in type 1c GSD is not known. There is evidence (Chen *et al* 1991) that

renal tubular damage may also be improved by corn starch diets to lessen the hypoglycaemia.

Another complication of hypoglycaemia is brain damage. It has been shown in experimental animals that to produce overt "brain damage" profound acute hypoglycaemia is necessary (Siesjo 1978). However it is likely that long-term or intermittent milder hypoglycaemia will produce more subtle changes in brain function - perhaps cognitive rather than focal motor or sensory changes. Cases 1 and 4 above were mentally obtunded and case 1's mental development was questioned several times (although never formally assessed). Children with type 1 GSD appear to show an adaptation to normal brain function at lower-than-normal blood glucose levels. Four children with type 1 GSD were shown to have a net uptake of lactate by the brain rather than net production which is the norm (Fernandes *et al* 1984). The suggestion therefore is that lactate can be used as a fuel by the brain in type 1 GSD. Normally ketone bodies are the secondary fuel for brain tissue, but type 1 GSD is characterised by a lack of ketosis. There is also animal work showing that transport of hexoses is increased across the blood brain barrier in animals (rats) exposed to chronic or intermittent hypoglycaemia (McCall *et al* 1986) suggesting that this may be another adaptive mechanism for the brain to derive enough glucose for normal function. There is no evidence that this mechanism occurs in type 1 GSD. The fact that most of our patients seemed to achieve normal growth and development may be related to the partial nature of their deficiencies resulting in less severe hypoglycaemia, or to the success of the adaptive mechanisms adopted by the brain to ensure a glucose supply.

As children with type 1 GSD approach and go through puberty there is a fall in the risk of developing hypoglycaemia (Greene *et al* 1981). The mechanism of this change is unclear but is associated with a rise in hepatic glucose output (Powell *et al* 1981, Kalhan *et al* 1982 and Tsalikian *et al* 1984). The timing of the change suggests a hormonal influence, however. None of the female type 1 GSD patients

previously described have reached the menopause when three of our patients reported worsening of their hypoglycaemic symptoms (despite unchanged dietary measures) which later improved with ovarian hormone replacement therapy. There have been short-lived trials of hormonal therapy (thyroxine and glucagon) for patients with GSD 1 (Koulisher & Pickering 1956 and Lowe *et al* 1962) which produced no clinical benefit. The data on the role of oestrogens above, while anecdotal as serial measures of glucose-6-phosphatase activity were not made, may indicate a benefit in giving oestrogen therapy to (female) type 1 GSD patients. The repeated study of glucose-6-phosphatase activity in man is virtually impossible because at present it requires repeated liver biopsies. I studied glucose-6-phosphatase activity in gut mucosa and circulating white blood cells (see below) with the aim of finding more accessible tissue to use. Further *in vitro* work is underway in Dr. Ann Burchell's laboratory to look at the effect of oestrogens on isolated microsomal rat glucose-6-phosphatase activity.

These cases described above strongly suggest a link between glucose-6-phosphatase activity and insulin release from the pancreas as has been postulated by Waddell & Burchell in 1989 following their observation of specific glucose-6-phosphatase activity in pancreatic islets. The possible controlling effect of glucose-6-phosphatase activity on cytosolic calcium levels (Benedetti & Fulceri 1986, Benedetti *et al* 1988 and Fulceri *et al* 1990) and the known role of calcium as a secondary messenger to stimulated insulin release (Colca *et al* 1983a & b, Rorsman *et al* 1984, Hellman 1985, Morgan *et al* 1987 and Nilsson *et al* 1987) are in favour of this association.

Case 6 had non-insulin dependent diabetes mellitus. The absence of the phosphate transport protein in his liver should have lead theoretically to hypoglycaemia as in the other reported cases of a type 1c GSD. The first reported type 1c patient was an insulin-dependent diabetic girl (Nordlie *et al* 1983) who had symptomatic hypoglycaemia unrelated to insulin dosing. Since then other type 1c cases have been described (eg Burchell *et al* 1989) and all the type 1c cases except case 6 above

have been hypoglycaemic at times. Why case 6 did not have hypoglycaemia is unclear. The pattern of measured microsomal glucose-6-phosphatase activity in case 6 is not that of uncontrolled diabetes mellitus with increased latency for glucose-6-phosphate and an increase in the Vmax in disrupted structures. The mechanism of hypoglycaemia in type 1c GSD is postulated to be phosphate accumulation in the lumen of the endoplasmic reticulum (as phosphate transport to the cytosol is non-existent) inhibiting the activity of the catalytic subunit (Arion *et al* 1980).

Case 7 had impaired glucose tolerance and a very unusual pattern of glucose-6-phosphatase deficiency previously only seen in premature infants (Burchell 1990 and Burchell & Gibb 1991). The pattern of "pseudo"-type 1b glucose-6-phosphatase abnormality demonstrated in case 8 has similarly been seen occasionally in hypoglycaemic infants, and the hyperinsulinism this case showed is further support for a role for glucose-6-phosphatase in insulin release from the pancreas. However because case 8 responded so well to a single dose of a somatostatin analogue it is tempting to postulate that the underlying defect in his pancreatic islets is a deficiency of or insensitivity to native somatostatin. The nature of the inhibitor of the hepatic microsomal glucose-6-phosphate transporter in case 8 is unclear, but may possibly be a peptide related to insulin as such high levels of this protein were measured in response to glucose loads.

Several defects in islet cell hormone release have been observed in other such patients with postprandial hypoglycaemia (including hyperinsulinism and blunt glucagon counter-responses) and some of these patients have responded biochemically to somatostatin administration (Hadji-Georgopoulis *et al* 1980, Foa *et al* 1980, Lev-ran & Anderson 1981 and Baschieri *et al* 1989). It is possible that at least some of these patients had an identical or very similar glucose-6-phosphatase abnormality as case 8 above.

Some of the observed glucose-6-phosphatase abnormalities described here may have been acquired - cases 6, 7 and 8 all had lymphocytic infiltration seen on routine histology of their liver biopsies suggesting an ongoing pathological process. The mechanisms of such acquisitions are very unclear and must be purely speculative. In case 6 one would have to postulate a defect developing at the gene controlling the production of the T2 protein, how common this is is unknown as presumably other type 1c GSD patients are asymptomatic of hypoglycaemia as this man was and therefore are not diagnosed. Case 7 would have to have a defect controlling the regulation of production of all the proteins of the glucose-6-phosphatase system. The presence of IgG to the Epstein-Barr virus is probably irrelevant as this is a common finding in a healthy population and infection with this virus does not cause hypoglycaemic symptoms. However it would have been interesting to make serial measures of glucose-6-phosphatase activity in case 7 to see if her glucose-6-phosphatase activity returned to normal. The abnormality in case 8 is the most obviously acquired of the three, but the nature of the inhibitor to T1 and the pathogenesis of this inhibitor are as yet unclear.

The glucagon screening test as described and interpreted here I suggest is a very important tool in the assessment of patients with hypoglycaemia. Without this test 6 of the 7 cases above who had the test done would not have had a correct diagnosis made (case 6 is the only one where the test was normal). Most of the cases above had symptoms suggestive of hypoglycaemia, but the hypoglycaemia was very difficult to prove biochemically. There has been almost an epidemic (especially in the USA) of patients describing symptoms suggestive of hypoglycaemia such that some authors have questioned the diagnosis as an entity at all (Ford *et al* 1976, Nelson 1985, Anderson & Lev-ran 1985 and Betteridge 1987). Often the symptoms these patients describe could be functional and in the absence of demonstrable hypoglycaemia even by prolonged fasting and exercise, a "functional" diagnosis is even more likely to be made by the physician. However, the addition of a fasting

glucagon test as described here to the investigation of such patients will hopefully mean that more of these subjects are correctly diagnosed. Of course the glucagon test is not in itself diagnostic and must be combined with the careful examination of a liver biopsy in a laboratory which is used to dealing with such samples and is prepared to assay the samples with three substrates to ensure a correct diagnosis (glucose-6-phosphate, pyrophosphate and mannose-6-phosphate) - see Burchell 1990 and Burchell & Gibb 1991.

Case 8 demonstrated hypoglycaemia after his glucagon challenge as did the majority of cases in a 'pooled' series of type 1b GSD patients (Ambruso *et al* 1985). Case 8 had a "functional" type 1b glucose-6-phosphatase system defect, but did not have a true type 1b GSD - a deficiency of the glucose-6-phosphate transport protein. It would be interesting to study other so-called type 1b GSD patients with more refined assay techniques than the freeze/thaw method of decreasing the glucose-6-phosphatase latency which is most commonly used to diagnose type 1b GSD, in order to see if they also have inhibitors of T1. The freeze/thaw method of diagnosing type 1b GSD does not allow measures of microsomal intactness on fresh or thawed tissue as do our methods of assaying with mannose-6-phosphate and using histone to remove the latency of the glucose-6-phosphatase system. This assessment of microsomal intactness is very important in interpreting glucose-6-phosphatase activity data. Once the microsomes have been frozen and thawed it is then difficult to know the intactness of the microsomes and there will have been some loss of glucose-6-phosphatase activity by the process of freezing and thawing.

There are limitations to the glucagon challenge I have described. A blunt blood glucose response is not diagnostic of a glucose-6-phosphatase abnormality: we have described two women (a mother and daughter) who had symptoms suggestive of hypoglycaemia, blunt glucagon tests and hepatic phosphorylase kinase deficiency (Appendix C and Pears *et al* 1992). Also we did not perform liver biopsies (for ethical reasons) on people with symptoms suggestive of hypoglycaemia who had

"normal" glucagon tests by the criteria we adopted. This is a further argument for the use of a more accessible tissue such as blood cells or gut mucosa to study glucose-6-phosphatase activity. The interpretation of the glucagon test in adults is open to question. We used the criteria described by Fernandes *et al* (1969) for children rather than the more recent recommendations of Dunger and Leonard (1982). Had we used the latter criteria (described to identify complete type 1 GSD cases in children) then 4 of the above cases who were given a glucagon test would have had a "normal" response; subsequent liver biopsy would not have been performed and a correct diagnosis would not have been made. A rise in blood glucose of ≤ 4 mmol/l is also arbitrary, but was specific for the group of patients described above. To establish a normal response to glucagon in adults it would be necessary to biopsy all patients given a glucagon test or it would be perhaps more ethically acceptable to perform glucagon tests on all patients undergoing liver biopsy. However these individuals presumably have a liver abnormality requiring a biopsy and this may alter the blood glucose response to glucagon. Best of all would be to study glucose-6-phosphatase activity in all patients with symptoms suggestive of hypoglycaemia and try to establish a normal glucagon test response in adults. As mentioned this would practically require examining glucose-6-phosphatase activity in blood cells.

The routine histological examination of liver samples from these patients proved to be of little benefit. The histological changes appeared to be very non-specific and the glycogen often appeared to leach out of the tissue samples during preparation. Careful preparation of samples of liver for electron microscopy revealed more abnormalities and gave a better measure of glycogen stores. However the biochemical assessment of the glycogen content is undoubtedly the most accurate.

The treatment of type 1 GSD is primarily dietary (see Fernandes *et al* 1988 for recent review) and the diet differs at different ages. In severely affected infants this can involve continuous night-time feeding via a nasogastric tube/gastrostomy.

Portacaval shunting initially showed some benefit (Corbeel *et al* 1983) but has since been abandoned as the beneficial effects have been shown to be temporary: liver transplantation (performed for hepatocellular carcinoma in individual cases in specialised centres) has been reported to improve growth and well-being (Kirschner *et al* 1991). Nasogastric feeding is not necessary now in older patients since the introduction of uncooked corn starch supplementation of the diet (Chen *et al* 1984, Smit *et al* 1984 and Fernandes *et al* 1988). Uncooked corn starch is slowly broken down to glucose in the gut over a period of hours providing a "depot" of glucose for absorption. This was the dietary manipulation we used for the cases above. For most of our cases it produced a marked improvement in symptoms. In cases 3,4 and 5 dietary manipulation was supplemented by oral hormone replacement therapy. Case 8 required drug therapy to alleviate his severe symptoms, however his abnormality is different to the rest as it is not due to a structural defect in any of the proteins of the glucose-6-phosphatase system. Case 6 did not develop hypoglycaemic symptoms despite being on a carbohydrate-controlled, weight-reducing diet.

The long-term outcome of these described cases will be interesting, and the lessons learnt from them in terms of the diagnosis of such patients and what they have shown about the regulation, inter-relationship and physiological role of the glucose-6-phosphatase system are very important.

Summary

The detailed cases presented above provide valuable new insight into the clinical problem of hypoglycaemia and the benefit of using a glucagon test in the diagnosis of patients presenting with symptoms suggestive of hypoglycaemia. They show the benefit of the refinements in assay techniques described for studying the proteins of the hepatic glucose-6-phosphatase system and especially the need to assay each sample with at least 3 substrates (glucose-6-phosphate, mannose-6-phosphate and

pyrophosphate, each ± histone 2A). These cases also help to interpret previously published reports of cases of hepatic glucose-6-phosphatase deficiencies which in retrospect probably represent partial type 1 GSD patients.

Two of the cases presented here are unique (numbers 7 and 8) in the deficiency in the hepatic glucose-6-phosphatase system that was demonstrated. The abnormal glucose tolerance of cases 6, 7 and 8 suggest a physiological role for glucose-6-phosphatase in pancreatic islet cells in the hormonal responses to changing blood glucose levels. It is also possible to postulate that the glucose-6-phosphatase abnormality in these cases was acquired as they all had lymphocytic infiltration of the liver at biopsy. Case 8 had an unusual history in that he presented with reactive (or postprandial hypoglycaemia). However the hyperinsulinism following a glucose load, absent glucagon response to the subsequent hypoglycaemia and therapeutic benefit of somatostatin he demonstrated are identical to a recognised sub-group of patients with reactive hypoglycaemia, all of whom may have an inhibitor of T1.

The other recognised features of type 1 GSD patients (hyperlipidaemia, high blood urate and lactate levels, bleeding tendency, renal impairment and hepatic adenomata) were lacking in most of these cases, although wherever possible they are being followed to monitor their renal function and for the presence of hepatic tumours. The reasons behind the absence of the other abnormalities are not clear, but may simply indicate that the glucose-6-phosphatase activity was sufficient in each individual to maintain metabolic 'normality' at non-stressed times. The hyperuricaemia found in case 1 suggests that the glucose-6-phosphatase deficiency was more severe in this man, but case 4 also had no catalytic subunit protein identified by immunoblotting, but had normal plasma urate levels. The stimulatory effects of oestrogens on glucose-6-phosphatase activity can be postulated from the worsening of hypoglycaemic symptoms experienced by three of these patients at the time of the menopause, and the subsequent beneficial effect of hormone replacement therapy.

The need for easier access to glucose-6-phosphatase activity in man is shown clearly by these cases, both in screening people with symptoms of hypoglycaemia with "normal" glucagon tests and in follow up of the cases described. This is one of the major reasons for going on to perform the studies described in the next chapter.

CHAPTER FOUR

GLUCOSE-6-PHOSPHATASE IN HUMAN EXTRA-HEPATIC TISSUE

The liver is the tissue in which glucose-6-phosphatase is best characterised. However many organ systems are involved in type 1 glycogen storage disease (GSD) both directly by increased glycogen stores or indirectly with the complications (for reviews see Hers *et al* 1989 and Moses 1990 for examples). Unfortunately access to the liver is difficult and potentially dangerous. A needle biopsy is the safest technique, but itself still carries a significant morbidity and requires an overnight hospital stay in adults in whom the procedure is uncomplicated. Needle biopsy of the liver produces only a few milligrams of tissue, which in many centres is far too small to allow detailed analysis of enzyme activity and glycogen content. Even in Dr Ann Burchell's laboratory with expertise in microassays there is often frustration of full analysis by small sample size. Ethically it is impossible to repeatedly follow glucose-6-phosphatase activity in a subject using repeated liver biopsies performed just for this purpose. I was therefore interested in finding tissue more accessible than liver in which to study glucose-6-phosphatase activity. I was also looking to further the physiological understanding of glucose-6-phosphatase function(s) and explain some of the clinical abnormalities seen in type 1 GSD patients.

Glucose-6-phosphatase activity is claimed to have been demonstrated in a number of human extra-hepatic tissues (see Nordlie 1971 and Nordlie & Sukalski 1985 for reviews). As glucose-6-phosphate can be hydrolysed by a number of phosphatases, detection of specific glucose-6-phosphatase activity relies upon showing the presence of the characteristic 36.5 kDa doublet (or cross-reactivity with an

isoenzyme of differing molecular weight) on immunoblot analysis; glucose-6-phosphate hydrolysing activity with the kinetic characteristics of the hepatic microsomal enzyme; and inactivation of this hydrolysing ability by incubation at 37°C and pH 5 (Burchell & Waddell 1990b).

The presence of glucose-6-phosphatase in the kidney is well-established (Ashmore & Weber 1959 and Burchell *et al* 1988b). The physiological role of glucose-6-phosphatase in the kidney is unclear, however the enzyme is localised to the peritubular cells, suggesting a possible role in glucose transport. However glucose-6-phosphatase activity has been shown to increase in the kidneys of animals where renal gluconeogenesis is increased, such as after partial hepatectomy (Katz *et al* 1979).

Colilla *et al* 1975 also have shown low levels of specific glucose-6-phosphatase activity in pancreas, adrenals, brain and lung. Recent work using the detailed, sensitive assays described above and immunoblotting with antibody raised to the catalytic subunit have confirmed the presence of glucose-6-phosphatase in gall bladder mucosa (Hill *et al* 1989) and pancreatic islet cells (Waddell & Burchell 1988) as would be predicted by the data from cases 6, 7 and 8 presented in Chapter 3.

The mucosa of the small intestine is classically thought to be affected in type 1 GSD (eg Hers *et al* 1989), but this statement is rarely referenced in review texts reflecting, perhaps, the uncertainty surrounding the presence of the enzyme in gut mucosa. There are histochemical studies which claim to show the presence of the glucose-6-phosphatase enzyme in intestinal mucosa, but this data does not take into account the presence of non-specific phosphatases (Hugon *et al* 1970 & 1971, Chabot *et al* 1978 and Calvert *et al* 1979). As yet there is no histochemical data on gut mucosa using the monospecific IgG raised to the glucose-6-phosphatase catalytic subunit protein.

There are reports of glucose-6-phosphatase activity in small intestinal mucosa of guinea pig, rat, mouse, and chicken (Ockerman 1964, Ashmore & Weber 1959, Colilla *et al* 1975, Anderson 1974 and Stritmatter 1972). However there are other reports that glucose-6-phosphatase activity is not present in rat intestinal mucosa (Ockerman 1964, Hers & DeDuve 1950 and Williams *et al* 1963). Similarly there are reports of the presence of glucose-6-phosphatase activity in human intestinal mucosa (Hers & DeDuve 1950 and Ginsburg & Hers 1960). Some workers have even claimed to show differences in intestinal glucose-6-phosphatase activity between subjects with type 1 GSD and their normal relatives suggesting that the diagnosis of type 1 GSD could be made from this tissue (Williams *et al* 1963 and Ockerman 1964). However none of this data allows for the presence of non-specific phosphatase activity, and none has used antibody shown to be monospecific to the catalytic subunit of glucose-6-phosphatase (Burchell *et al* 1988b).

There is a report of glucose-6-phosphatase activity in platelets (Linneweh *et al* 1962) and of serum glucose-6-phosphatase activity (Koide & Oda 1959), but in both of these reports non-specific phosphatase activity has not been allowed for.

The physiological role of glucose-6-phosphatase in extra-hepatic tissues is still unclear. The kidney has been shown to produce glucose by gluconeogenesis (see above). In the pancreatic islets the enzyme may play a role in hormone response to changes in blood glucose concentrations - this is supported by cases 6,7 and 8 above; and by the finding of glucose cycling in the pancreatic islets in mice (Khan *et al* 1989) which is increased in hyperglycaemic obese mice (Khan *et al* 1990). A role for glucose-6-phosphatase in controlling free intracellular calcium concentrations has been proposed (Benedetti *et al* 1985, 1986 & 1988). This latter role could explain the presence of the glucose-6-phosphatase enzyme in a wide range of tissues which do not release glucose. The glucose-6-phosphatase stabilising protein is a calcium-binding protein and calcium is required for normal glucose-6-

phosphate transport (Waddell *et al* 1987b & 1990 and Chapter 5 below) so the inter-relationship between the two is complex.

I studied normal adult human intestinal mucosa and white blood cells with the aims of trying to explain some of the abnormalities seen in these tissues in type 1 GSD patients, to find a tissue more accessible than liver, kidney and pancreas in which to study glucose-6-phosphatase activity in man and finally to settle the controversy surrounding the presence of glucose-6-phosphatase in these tissues. The cases presented in Chapter 3 clearly demonstrate the difficulty of having to biopsy liver to study glucose-6-phosphatase activity, and that repeated study of the enzyme in man is impossible on ethical grounds.

4.1 GLUCOSE-6-PHOSPHATASE IN NORMAL HUMAN GASTROINTESTINAL MUCOSA

Many papers and reviews on type 1 GSD state that glucose-6-phosphatase activity is present in intestinal mucosa and that gut function is abnormal in this condition. This statement is never correctly referenced (see Hers *et al* 1989 for a recent example). There is an impression that some children with type 1 GSD have diarrhoea (Fine *et al* 1969 and Milla *et al* 1978) but the evidence for malabsorption is not convincing. In 1964 Ockerman demonstrated absent glucose-6-phosphatase activity in the jejunum of type 1 GSD patients compared to high levels in normal mucosa and in 1968 Lygre and Nordlie published data taken as showing small amounts of glucose-6-phosphatase activity in intestinal mucosa cells.

In this present study I aimed to show definitive evidence of specific glucose-6-phosphatase activity and the presence of the glucose-6-phosphatase catalytic subunit by immunoblotting in intestinal mucosal homogenates. If high levels of specific activity were found, I wanted then to assess whether intestinal mucosal biopsies could reliably be used to screen for glucose-6-phosphatase abnormalities. The use of this tissue would obviate the need for a liver biopsy in patients suspected of having

abnormal glucose-6-phosphatase activity. I have learnt that one centre in the United States routinely uses intestinal rather than hepatic biopsies to diagnose abnormalities of glucose-6-phosphatase (personal communication).

The main problems of the study were to safely obtain good tissue samples and to try and inhibit proteolytic degradation of the contents of the mucosal cells after homogenisation, while still retaining functional (for assay purposes) and structural (for immunoblotting) integrity of the glucose-6-phosphatase catalytic subunit. Macroscopically normal mucosal tissue was biopsied from all parts of the gut accessible to endoscopic scrutiny. The samples were treated as described in 2.5.1 and below. The samples used to assay specific glucose-6-phosphatase and non-specific phosphatase activities were treated more 'gently' to try and preserve enzyme activity. Tissue homogenates, centrifuged to remove nuclear and cell membrane debris, were used in place of microsomes to shorten the time (and so the risk of proteolytic damage) between sampling and assaying or denaturing. Tissue was not examined histologically to ensure its normality to minimise the number of biopsies taken from any one patient.

Glucose-6-phosphatase assays were performed using a modification of that described in 2.6.1(a). 80μl of only 30mM glucose-6-phosphate substrate containing histone 2A was used and the reaction was incubated at 30°C for 15 minutes to amplify the amount of phosphate produced. 20μl of tissue homogenate (diluted 1 in 4 v/v in SH pH7.4) was added to start the reaction. The non-specific phosphatase activity of the samples was obtained using the assay conditions for treatment at pH5 as described in 2.6.1(e). The specific activity (in μmol/mg/min) of the glucose-6-phosphatase enzyme in each sample was then calculated using the following formula:

$$\text{Specific activity} = \frac{(OD_{820} - \text{non-sp. } OD_{820}) \times \text{Dilution} \times 10}{\text{Factor} \times \text{mg protein added} \times 15}$$

Where OD_{820} = Absorbance at 820nm of the untreated homogenate assayed with 30mM G6P + histone

non-sp.OD_{820} = Absorbance at 820nm of the pH 5 treated homogenate assayed with 30mM G6P + histone

Factor = Correction factor for the glucose-6-phosphatase assay using liver homogenate (approx. = 2)

10/15 = Correction factor for the time of assay (15 rather than 10 minutes)

Results and discussion

The results of the assays are shown in Table 4.1 and two immunoblots are shown in Figure 4.1

The assay data show low specific glucose-6-phosphatase activity in mucosal cells from all parts of the intestine. The range of the measured activity from any one site was large and may represent inter-individual variation, but is more likely to be due to varying amounts of proteolytic damage to glucose-6-phosphatase. Further support for this comes from Figure 4.1 (a) which shows samples which had been used for measuring enzyme activities separated by SDS-PAGE and immunoblotted using antibody specific to the rat hepatic glucose-6-phosphatase catalytic subunit. The majority of samples have an apparent molecular weight significantly lower than the rat hepatic control samples. This was initially possibly thought to represent the presence of an isoenzyme of glucose-6-phosphatase in gut mucosa; however Figure 4.1(b) where the size of the catalytic subunit in liver and intestinal mucosa have the same apparent molecular weight makes this unlikely.

The specific activity of glucose-6-phosphatase measured in the mucosal samples is low compared to human liver (approximately 0.2 in the fed state). Data from other human tissue is lacking. This may reflect less enzyme activity, but is probably also a reflection of proteolytic damage.

Table 4.1 Measures of phosphatase activity in homogenates prepared from normal human intestinal mucosa sampled at endoscopy.
Specific and non-specific glucose-6-phosphatase activity were measured on the same sample from each site.

Tissue	Specific G6Pase activity ($nmolP_i$/min/mg protein)	Non-specific phosphatase activity ($nmolP_i$/min/mg protein)
Oesophagus	20	0
Oesophagus	60	30
Oesophagus	0	0
Oesophagus	15	0
Stomach	7.7	2.4
Stomach	7.3	0
Stomach	6.1	3.3
Duodenum	30	5
Duodenum	1	10
Duodenum	8.7	6.5
Duodenum	0	7.3
Ascending colon	9.9	0
Ascending colon	11	2.8
Ascending colon	22	6.2
Transverse colon	80	0
Transverse colon	6.6	7.6
Descending colon	7.4	3.9
Sigmoid colon	8	0

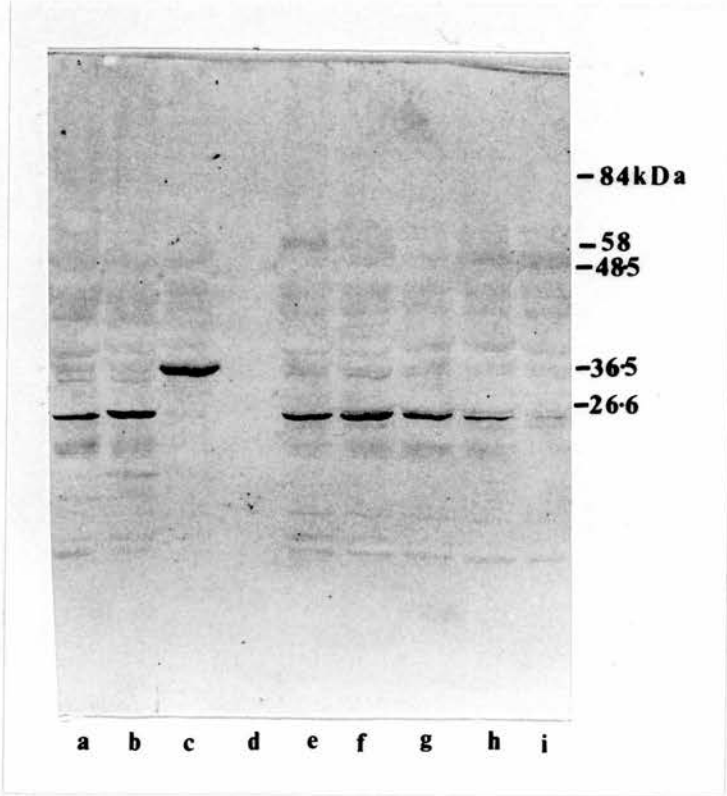


Figure 4.1 a

Immunostained Western blots of 7-20% SDS-polyacrylamide gels using antiglucose-6-phosphatase catalytic subunit antibody as the primary antibody.

(a) Plate shows samples of normal human intestinal mucosa collected for assay purposes (details in text). Lanes a,b & c contained 56, 64 and 20μg of gastric mucosal protein; lane d contained 34μg of duodenal mucosa protein; lanes e,f & g contained 54,6 and 20μg of colonic mucosa protein and h & i contained 46 and 36μg protein from sigmoid colon mucosa.

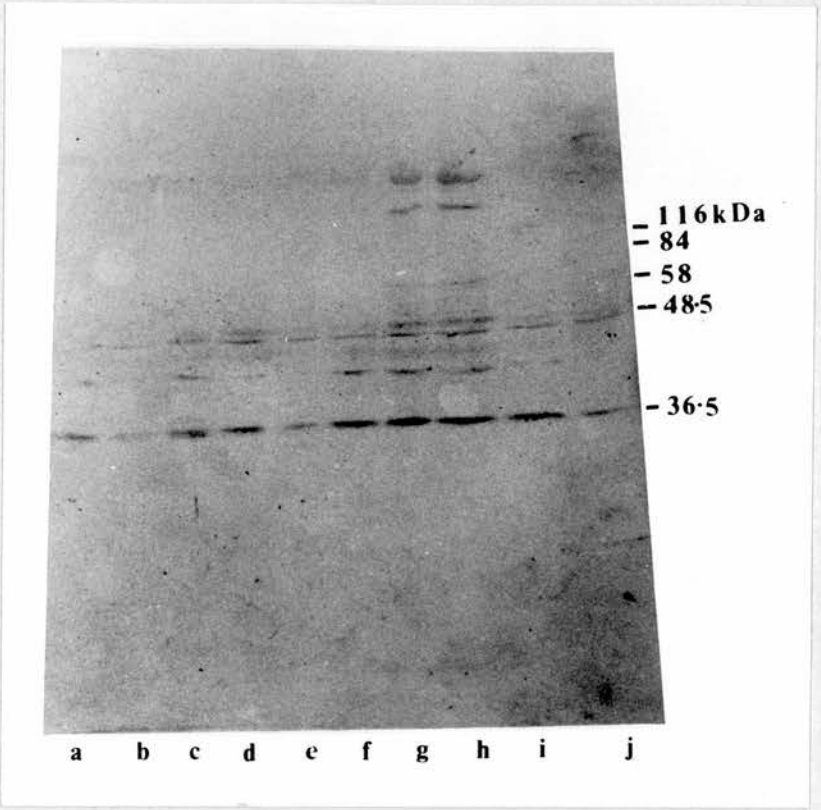


Figure 4.1 b

(b) Plate shows samples of normal adult human mucosa collected specifically for immunoblotting. Lanes a & j contained 9μg rat liver microsomes as control. All sample lanes were loaded with 50μg of protein.Lane b - oesophagus; c & d - stomach (different subjects); e - duodenum; f - ascending colon; g - transverse colon; h - descending colon; i - sigmoid colon.

The blot shown in Figure 4.1 (b) is from samples specifically collected for running on gels (see 2.5.1) and shows the enzyme band in all samples to have the same apparent molecular weight as the rat liver controls.

The glucose-6-phosphatase activity of the samples collected specifically for running on the gels was not tested as these samples were collected into a large number of protease inhibitors which would themselves interfere with the assay and the samples when spun were placed immediately into boiling SDS and mercaptoethanol after homogenisation and centrifugation.

These data presented above indicate the presence of glucose-6-phosphatase in mucosa cells at all levels of the normal adult gut. The measured specific glucose-6-phosphatase activity is low in all parts of the gut compared to the liver and shows inter-sample variability at the same level of the gut. Much of this variability is due to proteolysis which occurred in my sample processing despite using careful measures to inhibit it. Therefore there is a danger of incorrectly diagnosing type 1a GSD from intestinal mucosa using assay data alone, false negative diagnoses would be less likely to be made if immunoblotting was performed too. Although type 1a GSD can occur in the presence of immunoreactive catalytic subunit (Pears *et al* 1992). The assay data presented here indicates that diagnosing type 1b and 1c GSD could not be diagnosed and subtle partial abnormalities of glucose-6-phosphatase such as those shown in Chapter 3 could not be proven from intestinal mucosa biopsies.

Previous studies on glucose-6-phosphatase activity in intestinal mucosa are consistent with my data (Lygre & Nordlie 1968 and Ockerman 1964) but the data in these earlier reports does not include immunoblot analysis of samples. Despite his assay, Ockerman "showed" the absence of glucose-6-phosphatase activity in jejunal mucosa from 2 cases of type 1 GSD compared to normal controls - while his methodology includes a method for assaying non-specific phosphatase activity the results presented on this non-specific data are sparse.

Diarrhoea is reported by some to be a common finding in patients with type 1 GSD (Fine *et al* 1969) especially after a glucose load where there is also increased glucose excretion in the stool (Milla *et al* 1978). The evidence for associated malabsorption is not convincing. There has been a report of abnormal barium enema findings simulating those seen in colitis in children with type 1 GSD who were either asymptomatic or had intermittent diarrhoea (Fellows *et al* 1975). This series however reported normal small bowel barium investigations on the same children. There are also reports of Crohn's-like colitis in patients with type 1b GSD (Roe *et al* 1986, Couper *et al* 1991 and Sanderson *et al* 1991). The link between type 1b GSD and Crohn's-like colitis is not clear but may be related to a neutrophil abnormality rather than to a defect in the intestinal mucosa. In Crohn's disease in otherwise 'normal' individuals disorders of neutrophil function have been described (for example Wandell & Binder 1982, Worsaae *et al* 1982 & Verspaget *et al* 1984) although the significance of these defects in the pathogenesis of Crohn's disease is not established.

The physiological role of glucose-6-phosphatase in intestinal mucosal cells is as yet speculative. In the stomach and duodenum it may be involved in handling of glucose absorbed from the lumen of the gut, but in the colon this is unlikely to be a normal physiological role although glucose can be absorbed from the rectal mucosa (this method can be employed to resuscitate hypoglycaemic diabetic children). The metabolism of mucosal cells isolated from several parts of the intestine has been studied and glucose appears to play a central role in providing energy for these cells in the fed state (Windmueller 1984 and Ardawi & Newsholme 1985). There are reports of increased glucose-6-phosphatase activity in mucosal cells isolated from the intestine of fasted rats compared to fed animals (similar to the situation in the liver), but these assays do not allow for non-specific phosphatase activity (Ardawi & Newsholme 1985). The proteins of the glucose-6-phosphatase system bind calcium (Waddell and Burchell 1987b and Chapter 5 below) and glucose-6-

phosphatase activity may help to control intracellular calcium levels (Benedetti *et al* 1988). Therefore the role of glucose-6-phosphatase may be the control of cytosolic calcium concentrations in intestinal mucosal cells.

4.2 GLUCOSE-6-PHOSPHATASE IN NORMAL HUMAN WHITE BLOOD CELLS

There were three reasons for studying white blood cells and neutrophils especially. Firstly they would provide a readily accessible tissue in which to study glucose-6-phosphatase activity under different metabolic circumstances and in which to diagnose glucose-6-phosphatase abnormalities. Secondly patients with Type 1b GSD are susceptible to bacterial sepsis and there are reports of abnormal neutrophil function in some of these patients (for example Anderson *et al* 1981, Narisawa *et al* 1983 & 1986, Seger *et al* 1984 and Bashan *et al* 1988). Finally there is at present no practical way to monitor changes in glucose-6-phosphatase activity in a single individual. This would be attractive for monitoring the metabolic response to therapy of type 1 GSD patients, diabetic patients (if a correlation between hepatic and blood cell glucose-6-phosphatase activity could be shown) and might make it possible to study the turnover times of the protein components of the glucose-6-phosphatase system.

None of the patients described in Chapter 3 had a history of recurrent sepsis, but case 1 did suffer from recurrent epistaxes as a child and poorly explained episodes of rectal bleeding. A bleeding tendency is well recognised in poorly treated type 1 GSD patients which improves with normalisation of ambient glucose levels. Case 3 had a factor XI deficiency but no clinical evidence of a bleeding tendency. The link between the partial type 1a GSD and factor XI deficiency is unclear and the two may have occurred coincidentally.

The development of malignant tumours in man can be related to defects in immune surveillance by lymphocytes: three of the patients described in Chapter 3 developed

malignant tumours (case 1 hepatocellular carcinoma; case 4 cervical carcinoma; and case 6 anaplastic small cell bronchogenic carcinoma). The last two are common cancers in the community and may be unrelated to the glucose-6-phosphatase abnormalities in these individuals. Hepatocellular carcinoma is a very common world-wide carcinoma especially in areas where the hepatitis B surface antigen is endemic and is a recognised complication of type 1 GSD, but is otherwise relatively uncommon in Britain. Three of the patients in Chapter 3 had lymphocytic infiltration on their liver biopsies such that two of them - cases 7 & 8 - were initially taken to have lymphomatous infiltrates of the liver. There is no data on an increased risk of developing tumours other than hepatic adenomata and carcinomata in type 1 GSD and only one other malignancy in a type 1b GSD patient has been described (acute myelogenous leukaemia - Simmons *et al* 1984).

In 1959 Koide and Oda published data interpreted as showing glucose-6-phosphatase activity in serum and different levels of this activity in different states. This data showed a lack of understanding of non-specific acid and alkaline phosphatase hydrolysis of glucose-6-phosphate and was later shown to be erroneous (Foz 1967). There is no specific glucose-6-phosphatase activity in serum.

Platelet abnormalities have been implicated in the bleeding tendency of some type 1a and b GSD patients (Corby *et al* 1974 and Ambruso *et al* 1985) and there has been a report of decreased glucose-6-phosphatase activity and increased glycogen stores in platelets from patients with type 1 GSD (Linneweh *et al* 1962). The measurement of phosphatase activity in this last paper did not result in specific glucose-6-phosphatase activity, but included non-specific phosphatase activity. The bleeding tendency in type 1 GSD patients is thought to resolve with dietary therapy (Czapek *et al* 1973) and is therefore thought to be secondary - possibly to the hypoglycaemia - and not due to an intrinsic platelet defect. However a bleeding tendency is not a feature of other hypoglycaemic syndromes, making this theory less likely.

Results and discussion

Blood was taken and the cells separated as described in 2.1.1 (d) and 2.5.2.

Initial work centred around immunoblot detection: if this sensitive technique had not shown the presence of glucose-6-phosphatase catalytic subunit then there would have been no reason trying to develop an assay to measure glucose-6-phosphatase activity in blood cells.

From the first cell separation procedure performed all blood cell types were separated by SDS-PAGE and immunoblotted. This was a preliminary experiment and did not use protease inhibitors. The resultant immunoblot using polyclonal IgG to the rat hepatic glucose-6-phosphatase catalytic subunit as primary antibody is shown in Figure 4.2. There was considerable non-specific staining, but the lane containing the separated neutrophils (lane 3) showed a doublet which ran alongside the rat hepatic microsomal control (lane 5). Platelets and monocytes were not studied further - this blot did not support the earlier claim of the presence of glucose-6-phosphatase in platelets (Linneweh *et al* 1962). Figure 4.2 also indicated that trying to separate whole blood by SDS-PAGE and then immunoblotting with anti-catalytic subunit primary antibody (lane 6) was not going to show clear protein bands, probably due to the large numbers of proteins loaded.

Subsequent studies were therefore confined to neutrophils. Yields of neutrophils from 10 ml of blood varied from 6.1 x 10^9/l to 67.2 x 10^9/l (n = 40) as measured in the 1 ml final suspension and were at least 91% pure as judged by the Coulter T540 automated cell counter. Aliquots of neutrophils were treated in various ways as described in 2.5.3.

Several combinations of protease inhibitors were tried to achieve immunoblots which did not show evidence of proteolysis (see 2.5.2). Proteolysis resulted in lower molecular weight peptides than the catalytic subunit being seen on

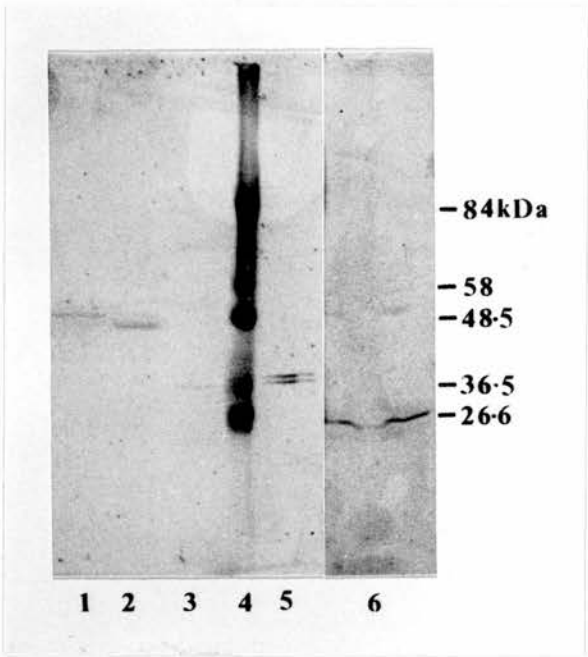


Figure 4.2

Immunostained Western blot of a 7-16% SDS-polyacrylamide gel using antiglucose-6-phosphatase catalytic subunit antibody as the primary antibody. All suspensions isolated as described from normal human blood with no protease inhibitors. Cell counts were not performed on these suspensions.

Lanes (1) 50μl suspension of platelets; (2) 50μl suspension of monocytes; (3) 50μl suspension of neutrophil polymorphonuclear leucocytes; (4) prestained molecular weight markers; (5) microsomes from starved rat liver as control (25μg protein); (6) 50μl normal human whole venous blood.

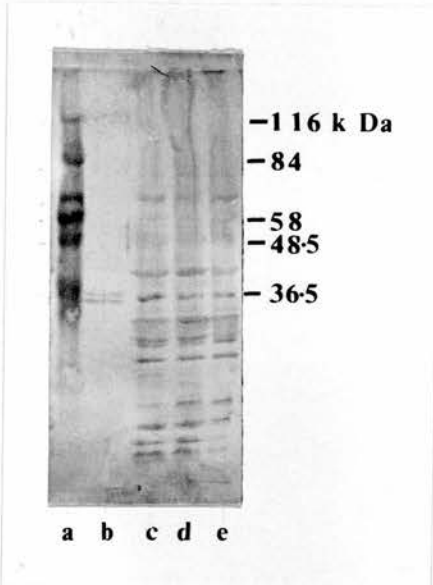


Figure 4.3

Western blot from a 7-20% SDS-polyarylamide gel using antiglucose-6-phosphatase catalytic subunit antibody as the primary antibody. Lanes (a) prestained molecular weight markers; (b) 25μg microsomal protein from starved rat livers; (c,d & e) contain dilutions of a preparation of neutrophils isolated from a normal human volunteer (me!) - numbers of cells contained were 22.4 x 10^5, 16.8 x 10^5 & 5.6 x 10^5 respectively. Preparation of neutrophils in protease inhibitors: PMSF, 1,10-phenanthroline, pepstatin A, trypsin/chymotrypsin inhibitor and aprotinin (details in text).

immunostaining of neutrophil samples compared to hepatic microsomal controls (Figure 4.3). Initially a combination of 1,10-phenanthroline and PMSF (2.5.2 [a]) was used and then other protease inhibitors were added as experience with the samples increased. The final combination (2.5.2 [c]) of sodium azide and PMSF was used as this had been shown to inhibit both proteolysis and the respiratory burst produced as neutrophils were disrupted (Gomez-Cambronero *et al* 1989). None of the combinations of protease inhibitors was able to alter the apparent proteolysis seen on immunoblotting, and none of them were better than using no protease inhibitors (Figure 4.4). Changing the buffer medium in which the neutrophils were finally suspended from Hanks to SH pH7.4 also made no difference to the apparent size of the catalytic subunit seen on immunoblotting.

It is possible that the apparent lower molecular weight of the glucose-6-phosphatase catalytic subunit from neutrophils compared to rat hepatic microsomal control is a true difference and that the neutrophil protein is an isoenzyme of the hepatic catalytic subunit or that the glycosylation states of the rat hepatic and human neutrophil catalytic subunit are different. However the presence of the low molecular weight peptides cross-reacting with the anti-catalytic subunit primary antibody such as in Figure 4.4 makes these suggestions less likely.

Attempts were made to assay glucose-6-phosphatase and non-specific phosphatase activities in neutrophil preparations, once the immunoblots showed firm evidence of the presence of glucose-6-phosphatase catalytic subunit. The cell preparations were assayed with only 30mM G6P containing histone 2A to detect specific activity as calculated from the equation in 2.6.1. After 15 minutes incubation of fresh, unfrozen neutrophil preparations very little phosphatase activity could be detected at all. Any measurable activity was small (see Table 4.2) and was often non-existent. The corresponding activity for fed rat hepatic microsomes (the least active rat liver preparation) was approximately 0.3. Modifying the assay conditions by adding more neutrophils and less stopping reagent to the assay in an attempt to amplify any blue

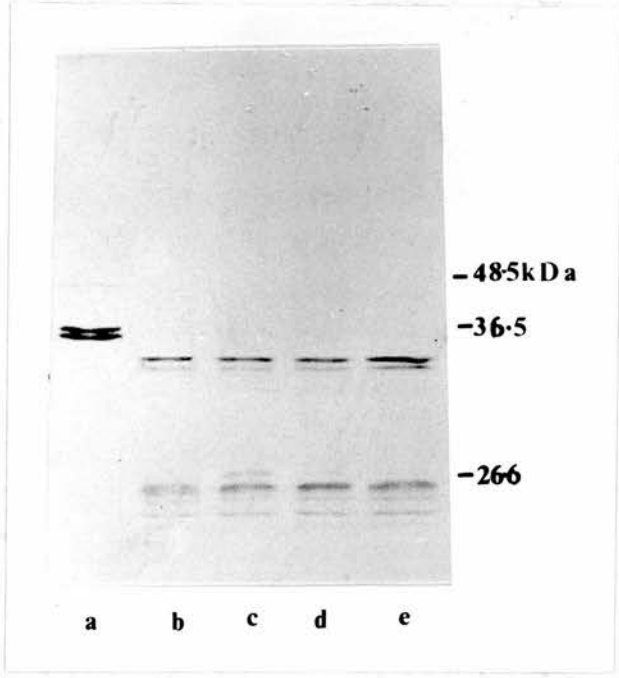


Figure 4.4

Immunostained Western blot of a 7-16% SDS-polyacrylamide gel using antiglucose-6-phosphatase catalytic subunit antibody as the primary antibody. Neutrophils in each lane separated from different normal human volunteers. No protease inhibitors used.

Lane (a) 25μg microsomal protein from starved rat livers; (b) 13 x 10^5 cells; (c) 16 x 10^5 cells; (d) 15 x 10^5 cells; and (e) 20 x 10^5 cells.

colour production (20μl of 120mM G6P substrate, assayed for 15 minutes with 80μl of neutrophil preparation before 500μl of stopping reagent was added) produced very high blank assay readings due to phosphate in the sample and no increase in measurable phosphatase activity. Preparing new substrates did not lower the blank assay readings and so this technique was abandoned.

Worse problems were encountered trying to measure non-specific phosphatase activity as the pH 5 treatment necessitated a 5-fold dilution of sample resulting in less detectable activity (Table 4.2).

Disruption of the neutrophil cell membrane prior to addition to the assay medium was attempted by several techniques - see 2.5.3. The rationale for this was to remove the additional barrier that the cell membrane provided to G6P entering the lumen of the endoplasmic reticulum before being hydrolysed. Assays performed with and without histone 2A in the absence of lubrol did not produce different measured activity implying that histone did not render the cell membrane permeable to G6P. Of the methods tried, mixing the neutrophil cell preparation with lubrol 2A (final lubrol concentration in sample, not assay, = 0.01% v/v) appeared to me to give the most consistent activity measurements but this was not confirmed by the meaned data (Table 4.2).

In summary the data from the immunoblotting is very suggestive of the presence of small amounts of glucose-6-phosphatase catalytic subunit in the neutrophil preparations. However the assay data, while it does not rule out glucose-6-phosphatase activity in neutrophils, is not conclusive of significant phosphatase activity of any nature. The most likely explanations for this are low amounts of glucose-6-phosphatase activity in neutrophils or proteolysis of the neutrophil contents during separation and/or assaying. The separation procedure for neutrophils is a lengthy one and while cells isolated by this technique are unactivated but functional (Ferrante and Thong 1980), it is possible that some degradation of internal proteins had occurred before the neutrophils came into

Table 4.2

Results of glucose-6-phosphatase activity measurements in samples of neutrophils isolated from normal human blood. Assays performed with 30mM glucose-6-phosphate + histone 2A as described in the text. Figures are glucose-6-phosphatase activity expressed as mg Pi produced / min of assay.

a) Normal assay.

Mean = 0.004 Range = 0.0 - 0.018 Number of assays = 17

b) Plus 0.01% v/v lubrol.

Mean = 0.005 Range = 0.0 - 0.018 Number of assays = 12

c) Plus 0.01% v/v lubrol, frozen at -70°C and thawed.

Mean = 0.007 Range = 0.0 - 0.033* Number of assays = 10

d) Samples treated at pH 5 prior to assay (1 in 4 dilution)

Mean = 0.006 Range = 0.0 - 0.003 Number of assays = 15

e) Samples diluted 1 part in 4 (no pH 5 treatment)

Mean = 0.00008 Range = 0.0 - 0.023 Number of assays = 15

* This value is much higher than any other obtained and is an error, but is included for completeness.

Values of activity are shown to 3 decimal places to emphasise the low measured phosphatase activity.

contact with protease inhibitors. Another problem with the assay could have been access of extracellular glucose-6-phosphate to the enzyme in the endoplasmic reticulum. However the neutrophils were completely disrupted (as shown by the Coulter T540 automated cell counter) by treatment with lubrol as described with or without freezing and thawing.

The protein bands shown by immunoblotting of neutrophil preparations are very strong and discrete, suggesting the presence of sufficient glucose-6-phosphatase catalytic subunit protein to allow assaying by the colourimetric method. This makes the theory of proteolytic damage the most likely explanation for the unmeasurable phosphatase activity.

I am not aware of a more rapid method for neutrophil isolation which reliably produces good yields of individual cell types, and as Figure 4.2 indicates immunoblot detection of the catalytic subunit of glucose-6-phosphatase on samples of whole blood is not possible due to large amounts of contaminating protein and the relatively small number of neutrophils in whole blood compared to, for example the number of red blood cells.

The physiological role of glucose-6-phosphatase in circulating neutrophils is speculative. Energy regulation within the cell and the need to produce glucose rapidly as a fuel for movement of the cell or for the ingestion and destruction of foreign material is a possibility, but a role in the control of intracellular free calcium is again likely.

There have been many studies of neutrophils isolated from type 1b GSD patients who are prone to bacterial infections, which have shown reduced numbers, mobility and function of neutrophils (Beaudet *et al* 1980, Anderson *et al* 1981, Gahr & Heyne 1983, Ambruso *et al* 1985, Shin 1990 and Moses 1990 for example). More detailed studies of normal neutrophils have shown that they use internal glycogen as a carbon source (Stossel *et al* 1970) and that calcium is an important intracellular messenger involved in glycogen breakdown, glucose transport and respiratory burst

activity (Snyderman & Uhing 1988). Neutrophils isolated from type 1b GSD patients show decreased glucose uptake (Bashan *et al* 1987 and Shin 1990), normal glycogen stores (Bashan *et al* 1988) but decreased chemotaxis (Beaudet *et al* 1980 and Anderson *et al* 1981) and poor respiratory burst activity (Kilpatrick *et al* 1990). These abnormalities described in neutrophils from type 1b GSD patients seems to relate to calcium mobilisation in the cells. Calcium mobilisation is essential for the respiratory burst in neutrophils (Heyworth & Segal 1986) and this calcium mobilisation is much lower in neutrophils from type 1b GSD patients (Kilpatrick *et al* 1990) when compared to normal controls and type 1a GSD patients who have functionally normal neutrophils. Neutrophils contain an isozyme of glucose-6-phosphate dehydrogenase in the lumen of the ER (Hino & Minakami 1982) which requires a glucose-6-phosphate transport system. If this glucose-6-phosphate transport system is the same as that used by glucose-6-phosphatase in type 1b GSD patients the function of this G6PDH isozyme will be impaired, thus further contributing to the abnormal neutrophil function.

This central role for the glucose-6-phosphatase system in intracellular calcium mobilisation (as well as glucose production) has been postulated for its role in other tissues (Benedetti *et al* 1985, 1986 & 1988, Fulceri *et al* 1990 and Waddell & Burchell 1988 and Chapter 3).

There have been three cases of Crohn's-like colitis developing in patients with type 1b GSD who also had demonstrable abnormalities of white blood cell (specifically neutrophil) function (Roe *et al* 1986 and Couper *et al* 1991). The pathogenesis of Crohn's disease in patients without type 1b GSD may in part be due to neutrophil dysfunction, and these observations are interesting in the light of the evidence presented above. Perhaps some patients with Crohn's disease have subtle glucose-6-phosphatase abnormalities such as are seen in type 1b GSD or inhibitors of T1 such as seen in case 8 in Chapter 3. The patient described in Chapter 3 with pseudotype 1b GSD (case 8) had no symptoms suggestive of intestinal disease, nor did he have

a history of recurrent sepsis. Neutrophil function tests were not carried out on this man.

SUMMARY OF DISCUSSION

The data in this chapter has proven the presence of specific glucose-6-phosphatase activity in normal adult intestinal mucosa at all levels of the gut. Even using the careful techniques employed, the level of measurable activity was too low and too variable (because of proteolysis) to make this tissue useful in studying human glucose-6-phosphatase activity. Therefore I would not recommend the use of intestinal mucosa cells to diagnose or monitor possible glucose-6-phosphatase abnormalities.

The presence of glucose-6-phosphatase activity in intestinal mucosa is in keeping with gut abnormalities being described in type 1 GSD patients (diarrhoea, clinical, histological or radiographic evidence of colitis). The role of the enzyme in this tissue may relate to local glucose production for cell metabolism, glucose transport through the intestinal mucosa cells and/or to regulating intracellular calcium concentrations.

Immunoblot analysis of neutrophils isolated from normal human blood indicates the presence of small amounts of the glucose-6-phosphatase catalytic subunit in these cells, but unfortunately it was not possible to establish an assay procedure which was able to show specific glucose-6-phosphatase activity. Further work is needed in this area, as the development of an assay for glucose-6-phosphatase in peripheral blood would make the study of the enzyme in man very much easier.

Future work involving the staining of tissue histochemically using the antibodies raised to the component proteins of the glucose-6-phosphatase system will make assessing the tissue distribution of the enzyme easier. Unfortunately this technique

will not give a functional assay, but would give an indication as to the relative amount of the proteins present.

Demonstrating the presence of the glucose-6-phosphatase system in neutrophils represents an important advance in understanding the physiology of these cells in health and disease. The role of glucose-6-phosphatase is probably not confined to controlling intracellular glucose / glucose-6-phosphate concentrations, but may well include the regulation of intracellular calcium concentrations in response to external stimuli. This is in keeping with demonstrated abnormalities of neutrophil function seen in type 1b GSD patients being due to an intrinsic cellular defect and not secondary to chronic hypoglycaemia.

CHAPTER 5

STUDIES ON THE MICROSOMAL GLUCOSE-6-PHOSPHATE TRANSLOCASE

5.1 INTRODUCTION

The cases presented in Chapter 3 clearly confirm the important role that glucose-6-phosphatase has in controlling blood glucose levels. Kinetic analysis of hepatic microsomal glucose-6-phosphatase shows that it is the transport of glucose-6-phosphate (by a transport protein) across the endoplasmic reticulum membrane from the cytosol to the lumen of the endoplasmic reticulum which is rate-limiting to the hydrolytic activity of the glucose-6-phosphatase system as a whole (Arion *et al* 1980b). Furthermore the capacity of the glucose-6-phosphate transport protein (T1) becomes more rate-limiting to the activity of the hepatic microsomal glucose-6-phosphatase system as the metabolic state of the animal changes from fed to starved to diabetic (Arion *et al* 1980b and Burchell & Cain 1985). Less is known of the regulation, structure and functioning of T1 than of any of the other proteins of the glucose-6-phosphatase system, despite its crucial position in hepatic glucose production. The role and key regulating position of T1 makes it an attractive prospect for pharmacological manipulation in hypoglycaemia (eg the type 1 GSDs and the cases in Chapter 3) or hyperglycaemia (eg diabetes mellitus where glucose-6-phosphatase activity and hepatic glucose production are increased). The effect of the inhibitor to T1 in case 8 described in Chapter 3 above which was to produce a fall in hepatic glucose production without apparently causing any of the other metabolic complications seen in type 1b GSD patients (Hers *et al* 1989 and Moses 1990 for reviews) supports this possible pharmacological site of action.

In 1982 a protein was identified which was thought to be T1 (Zoccoli *et al* 1982). Other workers then became hesitant about studying T1 as it was expected that this group would go on to describe the structure of T1, and then clone and reconstitute the transporter. However this information was not forthcoming and it now seems probable that the protein isolated by Zoccoli was not T1 but a sodium-independent hepatic transport protein for organic anions (Ananthanorayanan *et al* 1988).

The glucose-6-phosphate transport protein is one of many thousands of proteins which are associated with microsomal preparations, and the fact that by necessity it must be very lipid-dependent for its structure and function produces special difficulties when trying to isolate and then reconstitute it.

The aims of my studies on this protein were to try and isolate the T1 translocase, discover its biochemical regulation and whether it was possible to pharmacologically alter its capacity for glucose-6-phosphate transport.

5.2 MATERIALS AND METHODS

5.2.1 Studies on the T1 protein

Microsomes were prepared from livers of streptozotocin induced diabetic rats as described in 2.2 and 2.5. The postulated T1 protein was isolated by affinity chromatography as described in 2.7. An antibody to this protein was raised in a Cheviot sheep as described in 2.8.

To examine the effect of the antibody raised to an isolated protein on the glucose-6-phosphatase system, microsomes were mixed and incubated on ice with a given amount of IgG for 20 minutes before being assayed by the usual method with glucose-6-phosphate substrates without histone (after Waddell *et al* 1991). Pre-immune sheep serum was used as a control for these assays.

5.2.2 Studies on the effects of ions on microsomal glucose-6-phosphate transport

In order to identify an isolated protein as T1 it would be necessary to demonstrate glucose-6-phosphate transport by this protein. Before doing this it would be necessary to identify the mechanism of glucose-6-phosphate transport across the endoplasmic reticulum membrane. It seemed likely that glucose-6-phosphate transport is associated with the flux of other ions as glucose-6-phosphate transports as an anion. Sodium was the first cation studied.

A "low sodium" assay system for glucose-6-phosphatase /pyrophosphatase colourimetric assays and for radionuclide transport assays was developed to study the effect of cations on microsomal glucose-6-phosphate transport. Great care was taken to exclude as much sodium from the preparation of microsomes as possible. This was done by using the purest non-sodium salts available (for example the potassium salt of glucose-6-phosphate, pyrophosphoric acid, Aristar or Spectrosol grade salts of calcium, magnesium, cobalt, zinc and nickel chloride). When sodium chloride was added to this "low-sodium " system Aristar grade sodium chloride was used to minimise contamination with other cations. Milli-Q pure water (double distilled and purified through filters) was used to prepare all substrates and buffers and to wash all equipment (including assay tubes, tubes for storing microsomes and pipette tips) before use. EDTA was omitted from the colourimetric assay substrates as only the sodium salt was available. The concentrations of substrates used were 1, 1.4, 2 and 2.6 mM glucose-6-phosphate as at higher concentrations the Lineweaver-Burke plots became non-linear, presumably due to contaminants in the substrates reaching a sufficient concentration to affect the assay.

U^{14}C-glucose-6-phosphate sodium salt was mixed with an exchange resin (Dowex 50W-X8[H], BDH Chemicals) to exchange the sodium for H^+.

The sodium concentration of solutions was estimated where indicated using a Corning 410C clinical flame photometer.

5.2.3 Studies using amiloride hydrochloride

Once the requirement for sodium co-transport with glucose-6-phosphate became apparent a sodium transport inhibitor (amiloride) was used to try and modify glucose-6-phosphate transport.

Amiloride hydrochloride (Sigma Chemicals, Poole , UK) was prepared for use in assays by dissolving it in dimethylsulfoxide (DMSO) added dropwise at 46°C. The solution was then diluted to the required volume by the slow addition of SH pH 7.4 buffer (also at 46°C) and used fresh. The final concentration of DMSO in the colourimetric assay was 1%. 10μl of amiloride (or 1% DMSO to controls) was added to each assay tube at the preincubation stage and 10μl of microsomal dilution was then added. The final dilutions (v/v) in the assay were: fed rat liver microsomes 1 part in 75, starved rat liver microsomes 1 part in 100, diabetic rat liver microsomes 1 part in 150.

5.3 IDENTIFICATION OF A PEPTIDE: T1?

5.3.1 Isolation of a protein

In an attempt to isolate a protein with known affinity for glucose-6-phosphate, phlorizin and DIDS it seemed logical to use these compounds as ligands bound to Sepharose beads and utilise the technique of affinity chromatography as described in Chapter 2. Initially all three ligands were used: glucose-6-phosphate; phlorizin, a compound which binds to several proteins and selectively inhibits glucose-6-phosphate transport by T1 (Arion *et al* 1980a and Waddell & Burchell 1987a); and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), which is known to bind to several microsomal proteins and inhibits glucose-6-phosphatase activity at low concentrations in intact microsomes (Arion *et al* 1975 & 1980b, Speth & Schulze 1988 and Zoccoli *et al* 1980 & 1982).

The first affinity column used was that with Sepharose bound glucose-6-phosphate as ligand. Microsomes were initially solubilised by Triton X after treatment with concanavalin A to remove cell membrane debris. Fractions from the glucose-6-phosphate/Sepharose affinity column did not produce any protein bands when run on SDS-PAGE and tested for protein with Coomassie or silver stains. On one gel only a single protein band in all fraction and standard lanes was seen at 65-70kDa molecular weight. This probably represented contamination: the nature of the band is unclear, but it is a recognised feature of silver stains and it has been attributed to keratin proteins derived from the skin of the person preparing and running the gels (Morrissey 1981). More likely it was a contaminant, possibly microbial, in one of the buffer solutions used.

Both the glucose-6-phosphate/Sepharose and phlorizin/Sepharose affinity columns were run several times and did not produce any other peptides than the contaminant on silver-staining of SDS-PAGE gels.

In contrast the third run of the DIDS/Sepharose affinity column using lubrol-disrupted microsomes from livers of diabetic rats (see 2.7.1) produced positive Bradford reagent tests for protein in fractions 1 to 4 (fraction 1 was the strongest). It was also noted that lubrol produced a positive Bradford reaction, but the fractions collected came off the DIDS/Sepharose column before any TBS containing lubrol was added to it. The silver stained gel of these fractions showed several faint bands in the fraction lanes and a strong single band at an apparent molecular weight of approximately 66kDa. The Coomassie stain was blank. With every run of the DIDS/Sepharose affinity column the 66kDa peptide became more obvious and the band on the silver stain gel more discrete until the stains looked like Figure 5.3.1. The concentration of the peptide was highest in fraction 3 and decreased in subsequent fractions (fractions were 0.25ml) making the band unlikely to be a contaminant, but a peak of protein eluted early from the column.

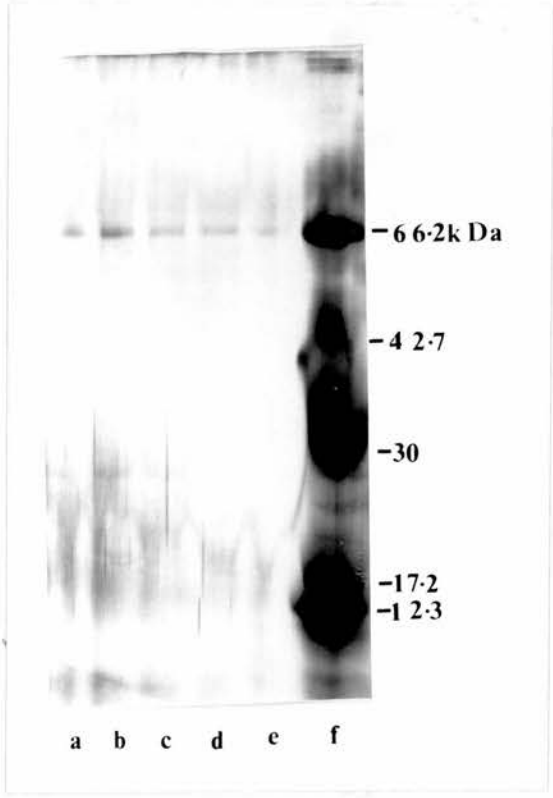


Figure 5.3.1

Silver stained 10% SDS-polyacrylamide gel. Lanes (a to e) each contained 50μl of fractions 1,2,3,4 & 5 respectively eluted from a DIDS/Sepharose affinity column loaded with microsomal protein from diabetic rat liver microsomes. Protein content of each fraction not determined.

Ten subsequent runs of this column were performed and all produced fractions which eluted early with 1M NaCl. After 6 runs other fragments of protein were demonstrated in the fractions after SDS-PAGE and silver staining of the gels. These contaminating fragments disappeared after the column was washed alternately in low (Na acetate, pH 4) and high pH (NaOH, pH9) to remove all protein bound to the column. A larger DIDS/Sepharose column was prepared and used on an FPLC system to produce larger quantities of protein.

When approximately 100μg of the protein had been isolated it was decided to raise an antibody to it in a sheep. Sheep are good at reliably forming antibody to injected protein, are relatively easy to care for and have a large enough circulating blood volume to allow repeated large volume sampling for serum separation. The isolated protein was concentrated by repeated treatments in a Speedivac system which centrifuges solutions in a vacuum at a temperature of 30°C. In the later stages of this concentration process, white cuboidal crystals were grown in the fractions which were taken to be sodium chloride as this was used to elute the protein from the column.

The decision to use the isolated protein to raise a polyclonal antibody was made in the hope that the antibody would be more specific than DIDS as a ligand when bound to Sepharose for isolating more of the protein for further study; the antibody could be added to assays to screen for any effects on glucose-6-phosphatase activity and could be used for immunoblot analysis of liver samples. The antibody could also be used to try screen proteins produced by, for example, a lambda-Zap library, sequence the protein and isolate the RNA and DNA encoding it.

Later I planned to try and reconstitute the protein into liposomes and demonstrate glucose-6-phosphate uptake by the treated liposomes as this is the best method for demonstrating the protein to be T1.

5.3.2 Studies with polyclonal IgG to the isolated protein

Three inoculations of equal size (approximately 30μg each) containing concentrated protein from the DIDS/Sepharose column fractions were injected into a Cheviot sheep, each one 4 weeks after the last.

Serum (10ml sample, then 400ml collection) was isolated from 4 weeks after the third injection and every 4 weeks after that until a total of 1200ml serum was taken. The serum was screened by immunostaining a dot-blot of microsomal protein and fractions from the DIDS column used to prepare the inoculate with the whole serum diluted 1 in 500 (v/v) in TBS as the primary antibody. The presence of a positive reaction in the relevant well was taken as proof of polyclonal antibody raised to the injected protein and a microsomal protein (Figure 5.3.2). The first 400ml of serum was treated with 90% ammonium sulphate as described in 2.8 to purify the IgG fraction. This was the antibody used to perform the rest of the studies and is called here anti-T1 IgG.

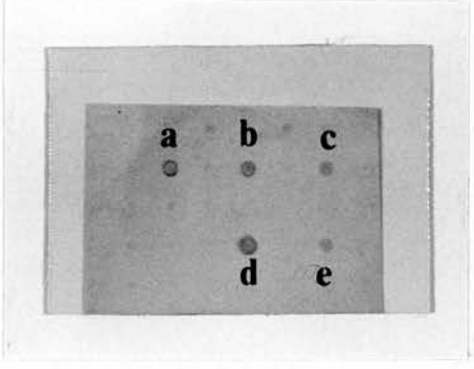


Figure 5.3.2

Immunostained dot blot using, as the primary antibody, untreated serum from an adult Cheviot sheep previously inoculated with protein isolated from diabetic rat liver microsomes by affinity chromatography with DIDS/Sepharose as ligand. Dots (a,b & c) contained protein from three fractions isolated by DIDS/Sepharose affinity chromatography known to contain the protein used for inoculation. The protein content of these fractions was not determined. Dots (d & e) contained 3.8mg and 2.9mg microsomal protein from the livers of diabetic and starved rats respectively.

a) Immunoblot analysis The anti-T1 IgG was used as the primary antibody (diluted 1 in 250 [v/v] in TBS) to immunostain blots of rat liver microsomal proteins separated by SDS-PAGE.

As shown in Figure 5.3.3 several major proteins were recognised by anti-T1. The fact that the microsomal proteins identified by the anti-T1 IgG were glycoproteins is clear from the shape of the bands which are shown - turned up ends and smearing running up the blot.

These microsomal proteins were at approximately 35kDa (about the same molecular weight as the glucose-6-phosphatase catalytic subunit [36.5kDa]); 45kDa which may be a biotin-binding protein in the microsomes (it was not found if a blot was "blocked" with biotin after milk - see 2.10.3) and is therefore recognised by the streptavidin/hydrogen peroxidase complex, not by the anti-T1 IgG. There was also a very strong protein band at an approximate molecular weight of 50kDa. However as Figure 5.3.3 shows the resolution of the molecular weight standards is poor and recently Amersham, one of the major producers of such standards, has published a warning that its molecular weight standards are not accurate even with good resolution on a gel. From a 7-16% gradient gel it is also not possible to determine the molecular weight of this heavily staining band accurately, although it is smaller than the 66kDa band originally isolated.

The fact that DIDS binds to glucose-6-phosphate binding sites and that a protein of molecular weight approximately 35kDa was identified with anti-T1 IgG by immunoblot analysis from a 7-16% acrylamide gel raised the possibility that the antibody was recognising the glucose-6-phosphate binding site on both T1 and catalytic subunit (if that is what the 50 and 35kDa proteins represent). However anti-T1 IgG did not show positive immunostaining with samples of purified catalytic subunit run on SDS-PAGE and blotted onto nitrocellulose. This was disappointing as if there had been cross-reactivity it would have been proof that the initial isolated protein was indeed part of the glucose-6-phosphatase system, but did not prove that

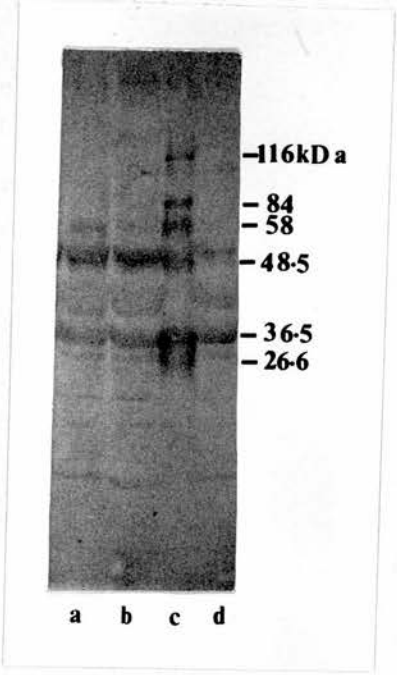


Figure 5.3.3

Immunostained Western blot of a 7-20% SDS-polyacrylamide gel using anti-T1 IgG as primary antibody. Lanes (a,b & d) contained 100μg, 60μg and 10μg of microsomal protein from livers of diabetic rats. Lane (c) contained prestained molecular weight markers.

anti-T1 IgG did not recognise the microsomal glucose-6-phosphatase catalytic subunit.

Equal amounts of protein from fed, starved and diabetic rat liver microsomes were separated by SDS-PAGE, blotted and immunostained with anti-T1 IgG (not shown). There was more protein (shown as darker, stronger bands) at both approximately 35 and 50kDa cross-reacting with the antibody in the microsomes from diabetic animal's livers than in the microsomes from starved and fed animals. If the protein at approximately 35kDa recognised by anti-T1 IgG is the glucose-6-phosphatase catalytic subunit this would be consistent with there being more glucose-6-phosphatase catalytic subunit protein in microsomes from livers of diabetic animals (Cain & Burchell 1985). However, the increased latency of microsomes from livers of diabetic animals has been suggested as indicating that there is no increase in number of T1 protein units in diabetes (Arion *et al* 1980b) rather than differing relative capacities for glucose-6-phosphate transport and hydrolysis. However if one of the two strong bands was T1 there was undoubtedly more of it in the diabetic microsomes (the band was darker).

When normal human liver homogenate was immunoblotted and stained using anti-T1 IgG (Figure 5.3.4) the antibody cross-reacted most strongly with the protein of approximately 35kDa - the 50kDa band was recognised, but less strongly. Many antibodies raised to proteins in one species do not cross react strongly with the same protein in another species; however the antibody to the rat catalytic subunit cross reacts well with the human protein which indicates strong preservation of sequence of the protein between the two species. It may be expected therefore, that the sequences of other proteins of the glucose-6-phosphatase system are also well preserved across species.

Unfortunately I did not get the opportunity to immunostain with the anti-T1 IgG on a sample of liver from a patient shown by standard kinetic analysis to have type 1b GSD as such patients are rare.

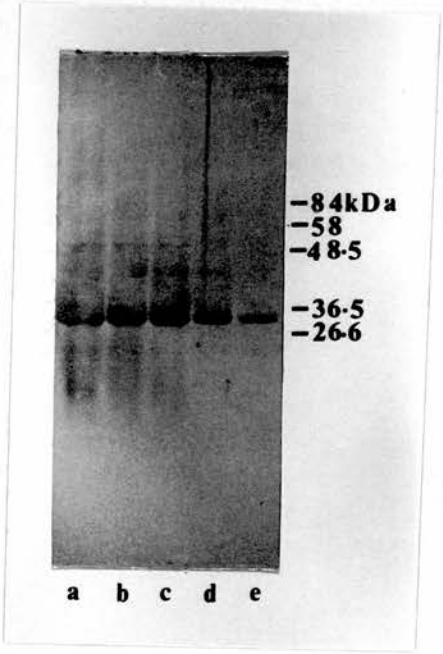


Figure 5.3.4

Immunostained Western blot of a 7-20% SDS-polyacrylamide gel using anti-T1 IgG as the primary antibody. Lanes (a to e) contained 80, 40, 24, 8, & 4 μg protein respectively from homogenate of normal human liver.

b) Affinity chromatography with anti-T1 Anti-T1 IgG was bound to Sepharose beads as described in 2.7. Lubrol solubilised diabetic rat liver microsomes were passed down a Sephadex G-25M column (to remove the lubrol) and then onto the anti-T1/Sepharose column. Protein was eluted from the anti-T1/Sepharose column (after 1 hour incubation at room temperature) with glycine.

Fractions from the anti-T1/Sepharose column were separated on 10% SDS-PAGE and then silver stained. As Figure 5.3.5 shows a number of protein bands were seen. This is a common finding with early runs of an affinity column where all non-specific binding sites on the Sepharose may not have been blocked. In later fractions one strongly staining band was seen at an approximate molecular weight of 66kDa (Figure 5.3.6) - the same apparent molecular weight as the original protein isolated by the DIDS column.

c) Assay data with anti-T1 Glucose-6-phosphatase activity in the presence of differing amounts of anti-T1 IgG was measured in microsomes prepared from starved and diabetic rat livers. The results of the assays performed are shown in Table 5.3.1. Time only allowed for a small number of "range-finding" assays to be performed hence the marked incompleteness of this data. No statistical analysis of the data has been performed as most of the figures in Table 5.3.1 are the mean of 2 assays only.

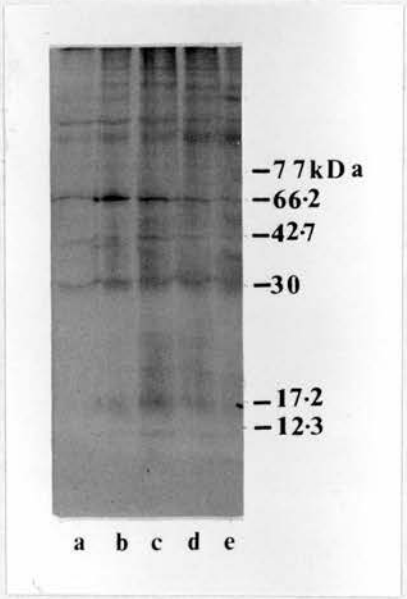


Figure 5.3.5

Silver stained 10% SDS-polyacrylamide gel. Lanes (a to e) each contained 100μl of fraction numbers 1,7,9,11 and 13 respectively eluted from the first run of an anti-T1/Sepharose affinity column loaded with microsomal protein from diabetic rat livers. The protein content of the fractions was not determined.

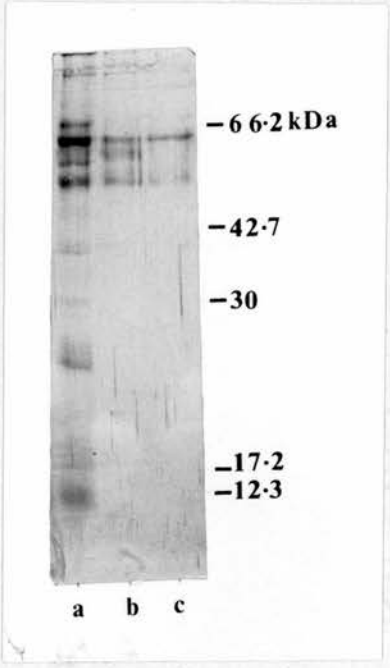


Figure 5.3.6

Silver stained 10% SDS-polyacrylamide gel. Lanes (a,b & c) contained 100μl each of fractions 8,12 and 14 eluted from a later run of the anti-T1/Sepharose affinity column than those in Fig. 5.3.5. The protein content of each fraction was not determined.

Table 5.3.1

Glucose-6-phosphatase activity measured in untreated rat liver microsomes preincubated with anti-T1 as described in the text. All substrates used did not contain histone.

	Starved rat liver microsomes		Diabetic rat liver microsomes	
Anti-T1 (μl)	Vmax (μmol/min/mg)	Km (mM)	Vmax (μmol/min/mg)	Km (mM)
0	0.26	3.0	0.40	4.9
20 p-is*	np**	np	0.42	3.4
5	0.27	3.1	0.41	4.2
10	0.24	3.0	0.39	4.1
15	0.23	3.0	np	np
20	0.22	2.9	0.38	3.5
25	0.22	2.9	np	np
30	np	np	0.37	3.2
50	np	np	0.33	3.1

* p-is = pre-immune serum

** np = not performed

No real conclusions can be drawn from these data. The anti-T1 IgG did not show an obvious effect on the glucose-6-phosphatase system in either type of microsome used.

The direct radionuclide assay of glucose-6-phosphate transport capacity (as described in 2.6.2) was not used as the biggest effect of anti-T1 IgG on microsomal glucose-6-phosphate transport should have been seen in microsomes from diabetic rat livers, where T1 capacity is most limiting to total glucose-6-phosphatase activity. It has not proved possible to perform this radionuclide assay using microsomes from diabetic livers as these microsomes rapidly block the nitrocellulose filter. Using nitrocellulose with a bigger pore size did not help this as a significant proportion of the microsomal protein appeared to not then bind to the filter but passed straight through into the effluent.

There is no reason why the anti-T1 IgG should affect T1 capacity. However if an obvious effect had been seen it would have been further confirmatory evidence that the antibody was indeed directed against T1. The antibodies to two other components of the rat hepatic microsomal glucose-6-phosphatase system do not affect the activity of these components - the anti-catalytic subunit and anti-phosphate/pyrophosphate transport protein (T2) antibodies have no inhibitory effect on their target proteins. The recently isolated antibody to T3/Glut 7 however does appear to inhibit the function of this glucose transporter (Waddell *et al* 1991)

5.3.3 Discussion

The presence of a single protein band at an apparent molecular weight of 66kDa on an SDS polyacrylamide gel when stained by the silver staining technique I used would be accepted by most workers as proving that a single protein had been isolated. This protein was probably a glycoprotein as shown by the up-turned ends of the band seen by immunostaining and the smearing running up the blot when immunostained with a polyclonal IgG (Figure 5.3.3).

However not all proteins take up silver stains when denatured in SDS gels, so it is possible that these fractions contained more than one protein. This may explain why a polyclonal antibody raised to these fractions recognised more than one protein on immunoblotting of microsomes. Alternatively the polyclonal antibody may be recognising similar amino acid sequences on more than one protein.

There is no proof that the 66kDa protein isolated by affinity chromatography with DIDS/Sepharose or either the 50 and 35kDa proteins then immunostained is the glucose-6-phosphate transport protein or even a component of the glucose-6-phosphatase system. There is some circumstantial evidence that the antibody raised to this protein is recognising at least one component of the glucose-6-phosphatase system (Figure 5.3.3). The cross-reactivity of the antibody raised to the 66kDa protein with a glycoprotein of approximate molecular weight 35kDa and the fact that by immunoblot analysis there is more of the 35kDa protein in microsomes from diabetic rat livers than in microsomes from starved and fed animals is compatible with the fact that there is more catalytic subunit protein (molecular weight 36.5kDa) in microsomes isolated from diabetic rats livers than from the livers of starved animals (Burchell & Cain 1985). DIDS binds to glucose-6-phosphate binding sites therefore it is possible that both T1 and catalytic subunit were bound by the DIDS/Sepharose affinity column. The glucose-6-phosphatase catalytic subunit protein dose not take a silver stain well and so may in fact have been present in the fractions. There is no evidence for increased amounts of T1 protein in diabetic rat liver microsomes, and Arion *et al* (1980b) claimed that the capacity of T1 is not increased in microsomes from diabetic rat livers compared to microsomes from starved animals: hence the increased latency of glucose-6-phosphatase activity in microsomes from diabetic animals.

It is possible that the original protein isolated from the DIDS/Sepharose affinity column and the larger of the two proteins strongly reacting to anti-T1 IgG on immunoblot analysis (Figure 5.3.3) represent the same protein and the apparent

difference in molecular weight is due to the immunoblots being performed on gradient gels, the inaccuracy of the molecular weight markers, differing glycosylation states of the proteins or any combination of these three reasons. However as suggested above, affinity chromatography using DIDS/Sepharose as ligand may have bound more than one protein component of the glucose-6-phosphatase system from solubilised microsomes but only one of which then appeared on silver stained SDS polyacrylamide gels. Countaway and Arion (1986) showed labelling of (at least) six hepatic microsomal proteins using tritiated DIDS and from this concluded that the purification of T1 could not be achieved using DIDS. However they also demonstrated that washing microsomes markedly increased DIDS binding sites (I deliberately did not wash the microsomes I used) and that DIDS bound covalently to the glucose-6-phosphate binding site.

Very recently work in Ann Burchell's laboratory on the rapid binding of ^{14}C glucose-6-phosphate to microsomal proteins has identified a protein of molecular weight approximately 35kDa (smaller than the glucose-6-phosphatase catalytic subunit). This work is, at the time of writing, very preliminary and is not published, however it agrees with my data above and would be compatible with the postulation that the fractions from DIDS/Sepharose binding did contain a protein which (as is the case with the catalytic subunit) does not silver stain. Antibody raised to these fractions recognised a microsomal protein of molecular weight approximately 35kDa (Fig 5.3.3) which may in fact turn out to be T1 and not the glucose-6-phosphatase catalytic subunit as I initially assumed. (In retrospect the early fraction lanes in Figure 5.3.1 may show a faint band at an approximate molecular weight of 30kDa).

The protein I identified by silver staining was larger than the 54kDa protein described by Zoccoli in 1982. This 54kDa protein isolated by Zoccoli *et al* using tritiated DIDS binding and which was postulated as T1 is not likely to be so. Zoccoli *et al* did not produce any subsequent corroborative evidence that the protein

they had isolated was the glucose-6-phosphate transporter. Countaway and Arion (1986) showed that this protein was not T1 and then Ananthanorayanan *et al* (1988) showed that it was likely to be a hepatocyte cell membrane sodium independent transporter for organic anions such as bilirubin and bromsulphthalein. DIDS cross-reacts with many microsomal and cell membrane proteins - especially anion transporters (Countaway & Arion 1986 and Cabantchik & Rothstein 1972 respectively). It is likely that Zoccoli *et al* had hepatic cell membrane contamination in their microsomal preparation. I was aware of the increase in microsomal DIDS binding sites shown by Countaway and Arion (1986) and so tried to treat the microsomes that I used as little as possible. It is encouraging that in my microsomal preparations very few proteins were recognised by the anti-T1 antibody - if the DIDS column had isolated a common protein or a cell-membrane protein it is unlikely that this pattern of 2 strong bands on immunoblot analysis would have been seen.

The definitive proof of any isolated protein being a microsomal transport protein would be the reconstitution of the protein into lipid vesicles and the subsequent demonstration of substrate (glucose-6-phosphate) uptake into vesicles which have had the protein incorporated into them. This kind of work is complicated and very time-consuming (see Racker 1985 for a review). It was felt that, at least initially, it would be worthwhile doing the simpler screening tests of the protein shown above using the polyclonal antibody raised to it in the sheep. Unfortunately time was a major limiting factor to completing even the initial work on identifying the protein. In retrospect it may have been better to use later batches of serum to produce IgG as the antibody production to the inoculated protein may have been greater. However none of the serum was disposed of and further work is under way with it and the proteins described above.

Subsequent studies on an isolated protein would be performed on the peptide isolated by the anti-T1 column, the peptide being eluted from the gel. It is unlikely

that a protein isolated in this fashion would be active, but the structure or sequence of the peptide could be found and antibodies more specific to the protein component of T1 could then be raised.

5.4 STUDIES ON THE MECHANISM OF GLUCOSE-6-PHOSPHATE TRANSPORT

5.4.1 Biochemical mechanism

In order to prove that an isolated protein was T1 it would be necessary to demonstrate glucose-6-phosphate uptake when the protein was reconstituted into liposomes. As well as having a protein it would be necessary to know the biochemical mechanism of glucose-6-phosphate transport. Also, in order to target pharmacological agents to manipulate T1 activity *in vivo*, a knowledge of this mechanism would be vital. Nothing was known at all of the mechanism of microsomal glucose-6-phosphate transport when this thesis started.

Arion *et al* (1980b) had suggested that glucose-6-phosphate transports across the microsomal membrane as a divalent anion. In order to maintain charge and pH neutrality on both sides of the microsomal membrane it would be necessary therefore that glucose-6-phosphate either cotransports with a cation or exchanges with another anion.

It was decided to investigate the former possibility, and to concentrate on sodium as the cotransported cation. The reasoning behind this decision was that transmembrane sodium transport is a common method of controlling intra- and extracellular milieu and sodium is not the predominant intracellular cation making it a logical ion to control transport across the endoplasmic reticulum membrane. The effect of sodium on microsomal glucose-6-phosphate transport had not been properly examined. DIDS is also recognised to interact with anion transport proteins

(Cabantchik & Rothstein 1972) and has a marked effect on T1 function (Arion *et al* 1978 & 1980b and Zoccoli *et al* 1980 & 1982).

To investigate the effect of sodium on hepatic microsomal glucose-6-phosphate transport it seemed logical to remove as much sodium as possible from the assay media and observe the changes in glucose-6-phosphatase activity.

When this "low sodium" system (prepared as in 5.2.2) was first used the effects on glucose-6-phosphatase activity, and especially glucose-6-phosphate transport seemed clear (Table 5.4.1). The Km of the intact microsomes rose markedly (implying decreased glucose-6-phosphate transport capacity) when assayed with potassium glucose-6-phosphate substrates compared to microsomes assayed with sodium glucose-6-phosphate substrates. This Km change was reversed by the addition of 1mM NaCl to the potassium substrate before the "low-sodium" microsomes were added. There was no significant difference in glucose-6-phosphatase activity between the "low sodium" and "normal" disrupted microsomes using sodium glucose-6-phosphate substrate.

Another difference between the "low-sodium" and normal assays was the absence of EDTA in the potassium salt substrates. This has been shown to not significantly alter glucose-6-phosphatase activity (Mithieux *et al* 1990). Assaying "normal" microsomes with potassium glucose-6-phosphate substrates showed virtually normal activity, also implying that potassium was not inhibiting glucose-6-phosphate transport or glucose-6-phosphatase activity (Table 5.4.1) in the "low sodium" system.

Table 5.4.1

"Sodium-free" microsomes assayed with substrates as indicated compared to "normal" microsomes.

(a) Microsomes from starved rat livers

Microsomal preparation	Substrate	Intact microsomes		Disrupted microsomes	
		Km (mM)	Vmax (μmol/min/mg)	Km (mM)	Vmax (μmol/min/mg)
Normal	Na G6P	3.3 ± 0.3	0.16 ± 0.01	0.5 ± 0.05	0.26 ± 0.02
Low sodium	K G6P	$7.9 \times 10^{8} \pm 7.6 \times 10^{8}$	$1.3 \times 10^{6} \pm 8.9 \times 10^{5}$	0.7 ± 0.1	0.24 ± 0.02
Low sodium +1mM NaCl	K G6P	3.4 ± 0.6	0.18 ± 0.02	0.6 ± 0.1	0.26 ± 0.01

(b) Microsomes from diabetic rat livers

Normal	Na G6P	5.2 ± 0.7	0.28 ± 0.05	1.6 ± 0.3	0.66 ± 0.07
Low sodium	K G6P	$1.2 \times 10^{9} \pm 4.3 \times 10^{8}$	$4.1 \times 10^{7} \pm 2 \times 10^{7}$	0.7 ± 0.1	0.53 ± 0.05
Low sodium +1mM NaCl	K G6P	6.6 ± 1.0	0.40 ± 0.02	0.76 ± 0.1	0.50 ± 0.1

Data are mean $\pm$ SEM of 4 assays (9 assays for the low sodium with no additive)

The range for the measured Km in both microsomal preparations using potassium glucose-6-phosphate substrates was large (8.1 to $6.9x10^9$mM for the starved and 8.7 to $3.5x10^9$mM for the diabetic microsomes). These numbers are meaningless as values; Kms as high as these are theoretical rather than a practical measurement, but the data do demonstrate quite clearly that in the "low sodium" system very little glucose-6-phosphate transport is occurring.

Microsomal preparations usually maintain their kinetic activity for around 3 months when stored at -70°C. These "low-sodium" microsomes, however started losing their activity after about 4 weeks storage at -70°C. I wanted to be sure that this data was reproducible. Therefore I prepared another batch of "low-sodium" microsomes from livers of starved rat livers. Only starved animals were used to prepare these microsomes as I wanted to perform radionuclide glucose-6-phosphate transport assays and it has not yet proved possible to perform this assay on microsomes from diabetic livers as they choke the nitrocellulose membrane. This choking blocks the filter meaning that the drainage of incubation mix is very slow or non-existent, but always unpredictable. In order to interpret the results of retained radionuclide in terms of glucose-6-phosphate transport it is necessary to know the time of incubation of microsomes with the radionuclide. Attempts were made with nitrocellulose with a larger pore size using microsomes from livers of diabetic animals, but the microsomal protein passed straight through the filter without binding.

The second preparation of "low-sodium" microsomes appeared identical to the first and the same buffers and salts were used (but freshly prepared). The intact microsomes again demonstrated a high Km when assayed with potassium glucose-6-phosphate, however this was not restored to normal by adding up to 20mM sodium chloride. This batch of microsomes appeared even less stable on storage at -70°C than the first batch as they started losing their activity within 2 weeks of preparation. Therefore a third batch of "low sodium" microsomes was prepared

from the livers of starved rats using the technique described above. This batch showed the by now expected high Km in intact structures using potassium G6P substrate which appeared "normal" in disrupted structures and when assayed with sodium G6P (and EDTA containing) substrates.

By this time I had stopped performing full kinetic analysis on each batch of microsomes with each additive. The 1mM glucose-6-phosphate substrate without histone was used to screen all microsomes and additives. When the measured Km increases, the activity at low substrate concentrations becomes very low.

The addition of up to 10mM sodium chloride did not reduce the high Km of low metal ion microsomes (ie increase the activity measured) assayed with 1mM KG6P. The effects of adding other anions than sodium to these "low sodium" microsomes was investigated (Table 5.4.2) and resulted in little or no increase in the activity with 1mM KG6P as substrate.

Table 5.4.2

The effect of adding other anions than sodium to "low sodium" microsomes prepared from livers of starved rats. All assay data presented is glucose-6-phosphatase activity (μmol Pi produced/minute) using 1mM KG6P as substrate.

Anion added	Activity	Range	Number of assays
nil	0.02	0.02-0.02	5
5mM KCl	0.03	0.02-0.04	4
5mM CsCl	0.02	0.02-0.02	3
5mM NH_4Cl	0.025	0.02-0.03	4

The addition of 1mM sodium acetate to the "low sodium" microsomes had the same effect as adding 1mM sodium chloride indicating that it was the sodium that was missing rather than an excess of chloride which was the underlying problem

(activity in the presence of 1mM Na acetate = 0.027 μmol/min and of 1mM NaCl = 0.02μmol/min: activity with 1mM Na G6P = 0.09μmol/min on the same preparation).

The addition of 5mM potassium chloride to "low sodium" microsomes prepared from starved rat livers showed no evidence of glucose-6-phosphate transport inhibition indicating that the lack of T1 activity was not due to potassium inhibition of the microsomes assayed with KG6P (activity with 1mM Na G6P = 0.07μmol/min and with 1mM Na G6P + 5mM KCl = 0.08μmol/min on the same preparation).

It was also clear that these "low sodium" microsomes were exhibiting extreme temperature instability (the glucose-6-phosphatase activity was decaying much faster at -70°C than in "normal" microsomes). As the glucose-6-phosphatase stabilising protein (required for normal catalytic subunit activity) is a calcium-binding protein (Waddell & Burchell 1987b) and the preparation of these "low sodium" microsomes involved the removal of all cations as far as possible except potassium, the additive effect of small amounts of calcium chloride to the "low sodium" microsomes was tried. The results of these assays are presented in Table 5.4.3.

Initially 1mM nitrilotriacetic acid (NTA) was added with the calcium in an attempt to control the concentration of free calcium in the assay (after Scott *et al* 1980). Waddell *et al* had already reported the effects of 100mM NTA on glucose-6-phosphatase activity - increased disruption of intact microsomes and inhibition of the catalytic subunit activity in disrupted microsomes (Waddell *et al* 1990). I hoped that these effects would not be seen at the smaller concentration of NTA (1mM) used here. From the data in Table 5.4.3 it is obvious that this hope was not justified, but more importantly it appeared that the addition of NaCl and $CaCl_2$ (even in microsomes which were losing their glucose-6-phosphatase activity in the presence of NTA) was sufficient to restore normal glucose-6-phosphatase activity to intact "low sodium" microsomes when assayed with potassium glucose-6-phosphate.

Table 5.4.3

Effect of assaying "low metal ion" microsomes prepared from livers of starved rats assayed with the additives and substrates indicated in the table.

a) 1mM K G6P as substrate:

Additive	Activity no histone (μmol/min)	Activity+histone (μmol/min)
Nil	0.058	0.179
NTA*	0.076	0.2
NTA + Ca^{2+}**	0.054	0.179
NTA + NaCl***	0.063	0.187
NTA + Ca^{2+} + NaCl	0.107	0.172

b) 1mM Na G6P as substrate

Additive	Activity no histone	Activity +histone
Nil	0.085	0.228

Key: * NTA = 1mM Nitrilotriacetic acid

** Ca^{2+} = sufficient $CaCl_2$ to have 1.41μM free Ca^{2+}

*** NaCl = 1mM NaCl

These microsomes will now, therefore, more properly be called "low metal ion" microsomes.

The amount of free calcium used in the above assays was determined by adding increasing amounts of calcium to 1mM KG6P and assaying the "low metal ion" microsomes in the presence of 1mM NTA. The amount of calcium which was the lowest to give the maximal increase in glucose-6-phosphatase activity was chosen. This was equivalent to a free calcium concentration of 1.41μM in the assay (see

Scott *et al* 1980) but does not allow for the non-specific binding of calcium by microsomes.

NTA was used in an attempt to control free calcium concentrations as there is a lot of non-specific calcium binding to microsomes and high calcium concentrations (mM) will cause clumping and precipitation of the microsomes.

From the results of the above experiments I designed a set of experiments to test the hypothesis that both sodium and calcium are required for microsomal glucose-6-phosphate transport. On the same day three different types of microsomes were prepared from starved Wistar rats. One preparation was of normal microsomes; another was of "low metal ion" microsomes as described above; and the third was of "low metal ion" microsomes prepared in the presence of 100μM calcium chloride in the 0.25M sucrose/5mM HEPES buffer. These microsomes were assayed on the day of preparation or were frozen immediately at -70°C and assayed within 48 hours. No NTA was used. The results of all these assays are shown in Tables 5.4.4 a, b and c. The concentrations of sodium and calcium chloride used in these assays were chosen to be deliberately excessive based on the assays with the earlier batches of "low sodium" microsomes. I was not, in these experiments, as much interested in the fine tuning of the relative concentrations of the two cations required as in the demonstration of a combined effect.

The data in Tables 5.4.4 a,b & c demonstrate clearly that the addition of millimolar concentrations of sodium chloride and micromolar amounts of calcium chloride to the "low metal ion" microsomal glucose-6-phosphatase system successfully restored the Km of intact microsomes to normal, but that the addition of just sodium or calcium chloride at these concentrations has a less marked effect. From the kinetic analysis of glucose-6-phosphatase activity of starved liver microsomes demonstrated first by Arion *et al* (1980a) the Km of intact microsomes is an indirect estimate of the capacity of the glucose-6-phosphate transport protein, T1.

Table 5.4.4

Results of glucose-6-phosphatase assays performed on microsomes prepared from starved rat livers on the same day and assayed fresh or after one freeze/thaw at -70°C within 48 hours of preparation.

a) Normal starved rat liver microsomes

Substrate	n	Additive	Intact microsomes		Disrupted microsomes	
			Km (mM)	Vmax (μmol/min/mg)	Km (mM)	Vmax (μmol/min/mg)
Na G6P	4	Nil	3.3 ± 0.5	0.16 ± 0.02	0.5 ± 0.1	0.26 ± 0.03
K G6P	5	Nil	6.6 ± 3.1	0.14 ± 0.05	1.4 ± 0.3	0.31 ± 0.03
K G6P	4	4mM NaCl	3.7 ± 1.9	0.12 ± 0.04	1.4 ± 0.8	0.31 ± 0.1

Table 5.4.4 continued

Glucose-6-phosphatase assay data on specially prepared starved rat liver microsomes.

b) "Low metal ion" starved rat liver microsomes

Substrate	n	Additive	Intact microsomes		Disrupted microsomes	
			Km (mM)	Vmax (μmol/min/mg)	Km (mM)	Vmax (μmol/min/mg)
Na G6P	3	Nil	4.4 ± 1.2	0.16 ± 0.04	0.6 ± 0.3	0.27 ± 0.03
K G6P	4	Nil	32.8 ± 4.2	0.61 ± 0.35	1.8 ± 0.7	0.29 ± 0.07
K G6P	3	4mM NaCl	13.3 ± 5.7	0.22 ± 0.03	1.5 ± 0.9	0.28 ± 0.03
K G6P	3	4mM NaCl + 100μM $CaCl_2$	6.3 ± 1.3	0.21 ± 0.05	1.5 ± 0.5	0.29 ± 0.03

c) "Low metal ion" starved rat liver microsomes prepared in the presence of 100μM $CaCl_2$

Substrate	n	Additive	Intact microsomes		Disrupted microsomes	
			Km (mM)	Vmax (μmol/min/mg)	Km (mM)	Vmax (μmol/min/mg)
Na G6P	3	Nil	3.2 ± 0.5	0.18 ± 0.02	0.6 ± 0.3	0.29 ± 0.03
K G6P	3	Nil	10.4 ± 2.7	0.24 ± 0.06	1.3 ± 0.1	0.27 ± 0.004
K G6P	4	4mM NaCl	4.5 ± 1.3	0.13 ± 0.05	2.0 ± 0.6	0.29 ± 0.05

All figures are mean ± SD of n assays.

Therefore the above data (Tables 5.4.4 a,b & c) suggests that these concentrations of sodium and of calcium are required for normal hepatic microsomal glucose-6-phosphate transport. The relative amounts of sodium and calcium that were required to restore normal glucose-6-phosphate transport capacity suggested that sodium was co-transporting with glucose-6-phosphate and that calcium was required to enable the microsomal protein(s) to transport both. The exact amount of both of these cations that are required is not clear from this data. The amount of free ionised calcium in the assay conditions here is impossible to estimate as there is significant non-specific binding of calcium by "normal" microsomes and presumably even more by "low metal ion" microsomes. It is possible that less than micromolar amounts of calcium are sufficient for normal microsomal glucose-6-phosphate transport. The glucose-6-phosphatase stabilising protein is a calcium-binding protein with an affinity for calcium in the micromolar range (Waddell & Burchell 1987b) raising the possibility that the stabilising protein is not only necessary for catalytic subunit activity but is also required for normal activity of the glucose-6-phosphate transport protein.

The data in Tables 5.4.4 b & c also shows that the Km of the disrupted microsomes is higher with potassium than sodium glucose-6-phosphate. This implies that the catalytic subunit has less affinity for the potassium salt. Perhaps there is potassium inhibition of glucose-6-phosphate binding to the catalytic subunit. This is a significant finding as it had been previously thought that the kinetics of microsomal glucose-6-phosphatase activity was the same irrespective of the salt of glucose-6-phosphate used as substrate.

In order to demonstrate that the above hypothesis of sodium co-transport with glucose-6-phosphate was correct the radionuclide assay of microsomal glucose-6-phosphate transport described in 2.6.2 was used.

As glucose-6-phosphate transport has been demonstrated across the microsomal membrane (Waddell & Burchell 1987) but sodium transport has not, initially

attempts were made to follow time-dependent sodium influx across the membrane of "normal" and "low metal ion" microsomes in the presence and absence of glucose-6-phosphate using lubrol-disrupted microsomes as controls. These assays were very inconclusive. Figure 5.4.1 shows the best and worst of these attempted assays. The background levels of sodium measured in both intact and disrupted microsomes was often high and masked any glucose-6-phosphate-induced effect.

It also became clear that in the presence of lubrol, mannose-6-phosphatase latency was not a reliable indicator of microsomal intactness. This proved to be due to detergent inhibition of mannose-6-phosphatase activity at a lubrol concentration much greater than 0.3% (see Figure 5.4.2). Following this discovery (which was made after the observation that mannose-6-phosphatase activity which was apparently low in the presence of lubrol did not increase in the presence of histone 2A) I used the increase in activity of the microsomal 1-naphthol glucuronosyltransferase as an assessment of microsomal intactness as this enzyme is much less sensitive to lubrol inactivation (see Figure 5.4.2).

I then compared the accumulation of ^{22}Na in intact microsomes before and after a 2 minute incubation in the presence of 1mM G6P and 4mM NaCl using, as controls, the same microsomes in the absence of G6P as control rather than detergent-disrupted microsomes. This was a truer control than the disrupted microsomes and it made the G6P-dependent sodium uptake more obvious. Figure 5.4.3 shows the results of these incubations in the three microsomal preparations described above: normal; low metal ion; and low metal ion plus 100μM $CaCl_2$. The graph shows the percentage ^{22}Na retained by the microsomes after 2 minutes incubation taking an incubation time of 15 seconds as 100%. (15 seconds after the start of the incubation was the shortest possible sampling time to allow for adequate mixing). Any significant sodium uptake by the microsomes in the first 15 seconds would amplify the differences shown in Figure 5.4.3.

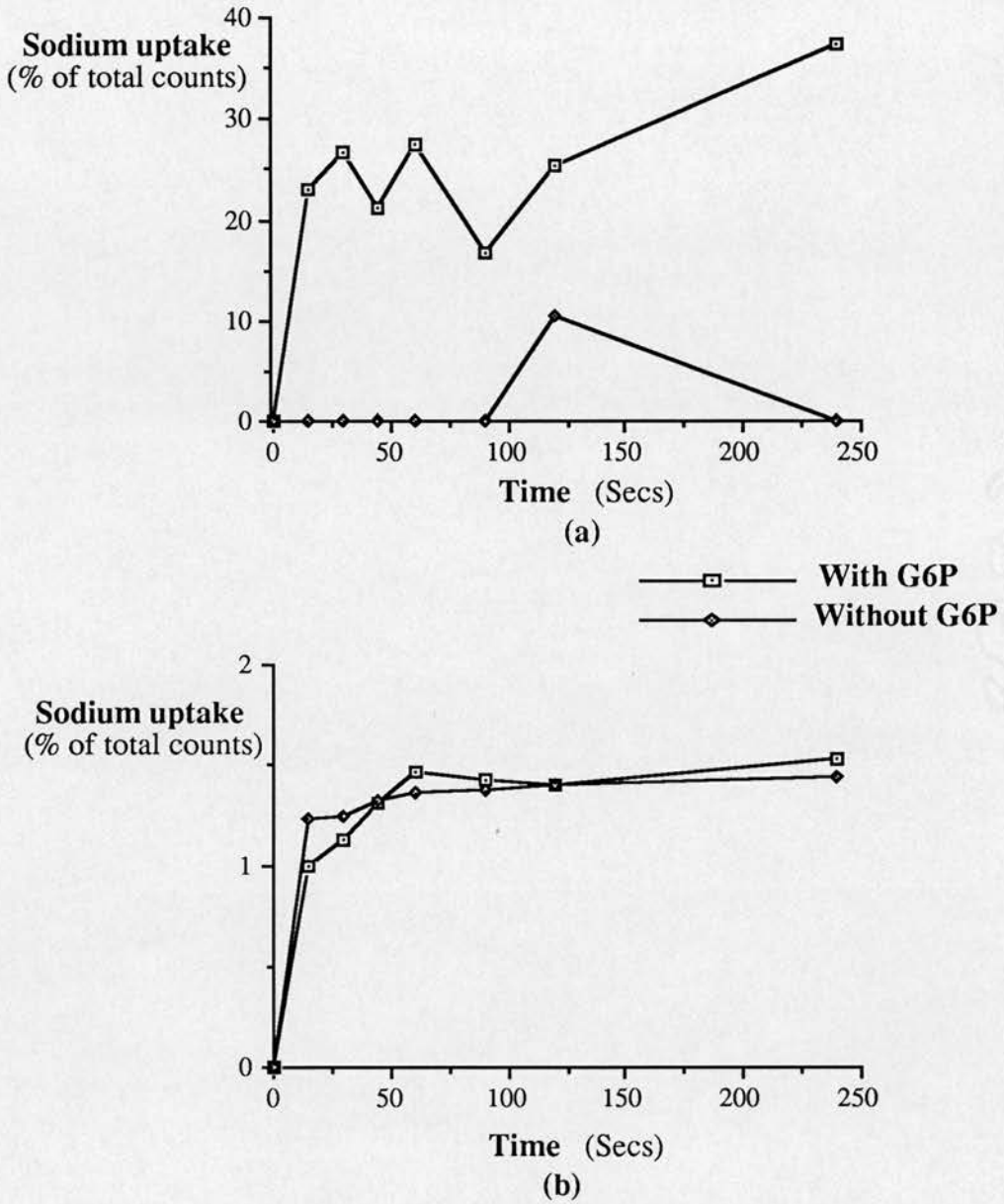


Figure 5.4.1

Results from two apparently identical assays following the glucose-6-phosphate-dependent flux of ^{22}Na into low metal ion microsomes as described in the text. Plate (a) clearly shows glucose-6-phosphate dependent uptake. However plate (b) shows high uptake of sodium even in the absence of added glucose-6-phosphate. The only difference between the two assays can only have been some contamination with cations of the assay system in plate (b).

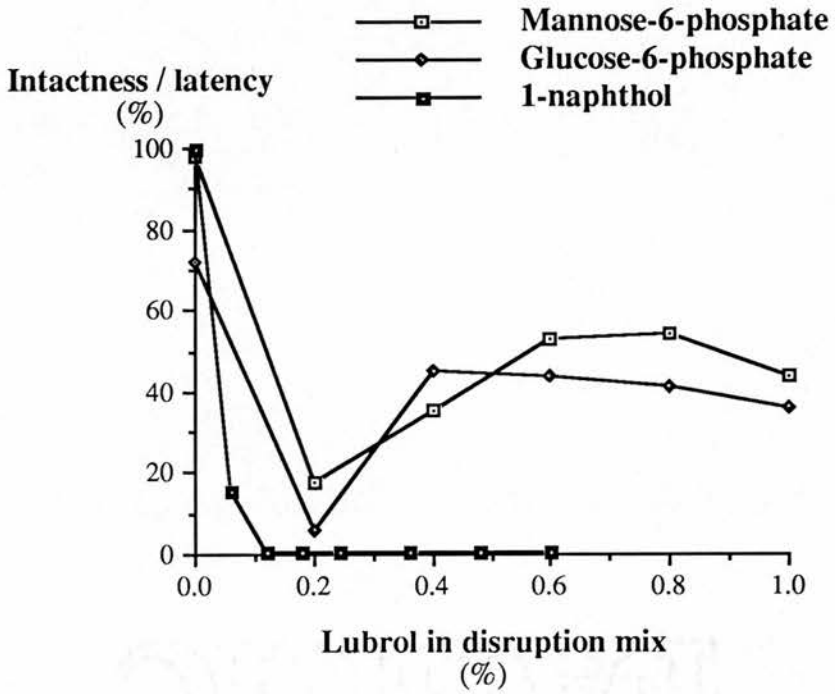


Figure 5.4.2

Measured intactness or latency of a single microsomal preparation after incubation of increasing detergent:protein ratios with three different substrates.

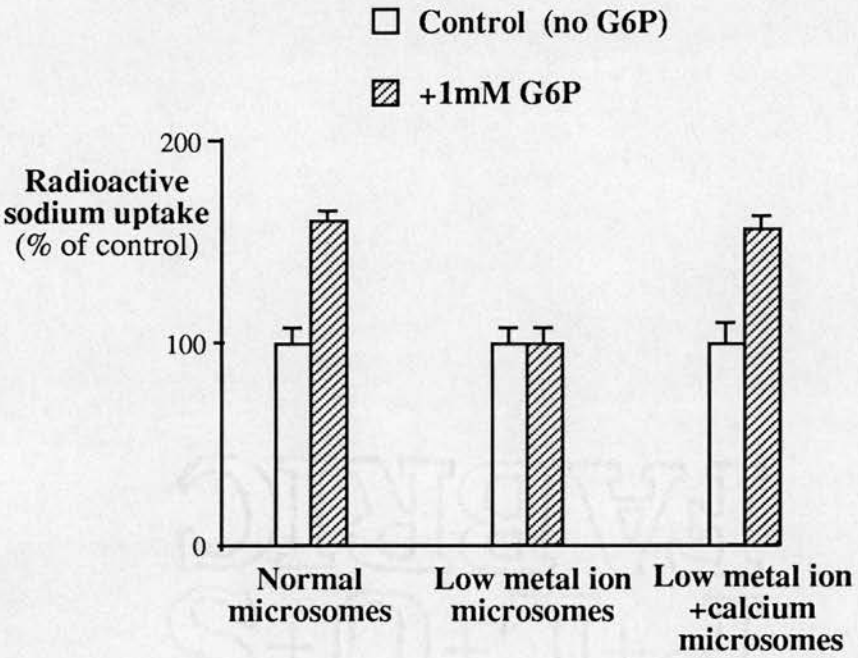


Figure 5.4.3

Results of assays measuring uptake of ^{22}Na by three different preparations of microsomes prepared from livers of starved Wistar rats. The clear requirement for calcium is shown. Details of the three microsomal preparations are given in the text. Results are expressed as a percentage of total ^{22}Na in the reaction mix using the uptake in control assays (no glucose-6-phosphate) as 100%. Data is mean $\pm$ SEM of at least 4 assays.

The effect of other divalent cations on microsomal glucose-6-phosphatase activity was examined using a single concentration (1.4mM) of glucose-6-phosphate and the colourimetric assay. 1.4mM glucose-6-phosphate was used as substrate so that low glucose-6-phosphatase activity could be more accurately measured. The results of these assays are shown in Table 5.4.5. The 1.4mM G6P substrate would also be more accurate than 1mM G6P at assessing falls in glucose-6-phosphatase activity but not too high that any change would reflect a Vmax effect rather than a Km one. The data in Table 5.4.5 shows that several divalent metal cations can affect the activity of both intact and disrupted normal and "low metal ion" microsomes using sodium and potassium glucose-6-phosphate substrates. A higher percentage value in Table 5.4.5 infers a lower Km and thus a greater affinity of the glucose-6-phosphate transporter or glucose-6-phosphatase catalytic subunit for the glucose-6-phosphate salt. The change in glucose-6-phosphatase activity was largest in intact microsomes using potassium glucose-6-phosphate as substrate and there was little difference between the metal cations used. However the data presented above and the data on the glucose-6-phosphatase stabilising protein make it likely that calcium is the cation involved *in vivo*. There was also little difference between the normal and low metal ion microsomes in terms of percentage increase in activity with the added metal ions (the absolute values were still very different). Why there is an increase at all in the glucose-6-phosphatase activity of normal microsomes is not clear. The affinity for the potassium salt of glucose-6-phosphate by T1 is lower than for the sodium salt and this may be overcome by the addition of small amounts of divalent cation. Alongside this it may be that metal ions themselves increase glucose-6-phosphatase activity, especially in the presence of potassium glucose-6-phosphate.

Table 5.4.5

Relative glucose-6-phosphatase activity with 1.4mM glucose-6-phosphate in rat liver microsomes from starved rats with different divalent metal cations added. Values are percentage of control glucose-6-phosphatase activity at 1.4mM G6P. All microsomes were more than 96% intact as calculated from mannose-6-phosphate latency.

a) Intact microsomes

Additive	Normal microsomes Sodium G6P	Normal microsomes Potassium G6P	"Low metal ion" microsomes Potassium G6P
Control	100	100	100
$CaCl_2$	94 *	279 ##	179 ##
$MnCl_2$	162 **	278 ##	217 ##
$MgCl_2$	149 #	225 ##	247 ##
$CoCl_2$	110 #	241 ##	252 ##
$NiCl_2$	97 *	254 ##	262 ##

b) Disrupted microsomes

Control	100	100	100
$CaCl_2$	90 *	129 ##	111 **
$MnCl_2$	104 *	119 #	116 ***
$MgCl_2$	94 *	137 ##	127 ##
$CoCl_2$	113 #	126 ##	122 ##
$NiCl_2$	89 *	130 **	137 ##

Key: * $p<0.5$ #$p<0.05$ **$p<0.005$ ***$p<0.01$ ##$p<0.0005$

p values calculated for each assay condition against the control value for that condition

The inter-relationship between hepatic microsomal glucose-6-phosphatase activity and divalent metal ions (especially calcium) is a complex one (see, for example Benedetti *et al* 1985 & 1987, Fulceri *et al* 1991, Mithieux *et al* 1990, van de Werve 1989 & Waddell *et al* 1990). Waddell did show that calcium (at high concentrations up to 10mM) increased T1 capacity in normal microsomes, but at the concentrations used had a more marked stimulatory effect on the activity of the T2 phosphate/pyrophosphate transport protein. At ambient calcium levels above 10mM microsomes clump thus a decrease in glucose-6-phosphatase is measured (Beaufay & de Duve 1954 and Kamath *et al* 1971).

The increase in glucose-6-phosphatase activity seen in disrupted microsomes with added cations and potassium glucose-6-phosphate as substrate is less than that in intact microsomes and probably reflects a decrease in affinity of the catalytic subunit for potassium glucose-6-phosphate which can be overcome by small amounts of added divalent cations. This change in activity was not seen in normal disrupted microsomes with sodium glucose-6-phosphate substrate.

As well as showing the importance of sodium and calcium ions in microsomal glucose-6-phosphatase activity, these data also indicate that the use of differing salts of glucose-6-phosphate will produce different glucose-6-phosphatase kinetics. Therefore workers should stipulate which salt they have used, or if directly comparing results this comparison should be done on data using the same salt as substrate.

5.4.2 Discussion

This data demonstrates for the first time the ionic dependence of glucose-6-phosphate transport across the hepatic microsomal membrane. It is important to understand this key step in hepatic glucose production and will make the assessment of the glucose-6-phosphate transport capacity of an isolated T1 protein possible.

Designing agents to pharmacologically manipulate T1 capacity could also be targeted at this.

Sodium co-transports with glucose-6-phosphate across the rat hepatic microsomal membrane and a divalent cation (probably calcium) is required for the normal transport of one or both. The data on sodium transport using trace amounts of radioactive sodium implied that this is not the only mechanism for sodium transport across the microsomal membrane.

The assumption made by Arion that glucose-6-phosphate transports as an anion appears correct. However in the same paper (Arion *et al* 1980b) it was suggested that sodium was not involved in glucose-6-phosphate transport. This statement was based on data showing a lack of change in glucose-6-phosphatase activity in intact rat hepatic microsomes by removing sodium from the buffers used to prepare the microsomes which were then assayed in substrates containing 20mM sodium salts as buffers of pH (and presumably other contaminating cations). The data above shows that 4mM sodium and only contaminant trace amounts of a divalent cation are sufficient for normal activity which explains Arion's error.

Sodium is the predominant extracellular cation with low intracellular concentrations, making it the logical cation to control transport across the endoplasmic reticulum and thus preserve electrostatic neutrality. The stoichometric ratio of sodium to glucose-6-phosphate transporting via T1 has not been determined: on a purely electrostatic basis it should be 2:1. No extrinsic source of energy was added (eg ATP or GTP), therefore the transport of glucose-6-phosphate is facilitative down a concentration gradient. Sodium presumably follows the glucose-6-phosphate to preserve ionic charge balance.

The data above does not demonstrate whether glucose-6-phosphate and sodium co-transport using the same or a different protein. The glucose-6-phosphate transport system of E. coli has been well characterised and DNA analysis has shown that it comprises at least 4 subunits (Weston & Kadner 1987). The evidence at present is in

favour of E. coli glucose-6-phosphate transport occurring down a phosphate concentration gradient rather than being driven by cation co-transport (Sonna *et al* 1988). If the mammalian system has developed from this then it is possible that the regulatory sodium transport system demonstrated above is a separate but closely linked protein to the glucose-6-phosphate transport protein.

Divalent metal cations decreased the Km of intact and disrupted microsomes (even normal microsomes) assayed with potassium glucose-6-phosphate. It seems that T1 and the glucose-6-phosphatase catalytic subunit have less affinity for potassium glucose-6-phosphate than for the sodium salt and divalent cations appear to overcome this decrease in affinity. The mechanism of this effect is unclear. Possible effects of the metal ions could be conformational changes in the glucose-6-phosphate binding sites or activation of "latent" molecules thereby changing the affinity of these molecules.

Previous studies examining the relationship between calcium and microsomal glucose-6-phosphatase activity have been confusing, largely, it has transpired because the effects of the buffers used. However recently it has been clearly shown that NTA (Waddell *et al* 1990) and EGTA (Mithieux *et al* 1990) will both affect glucose-6-phosphatase activity in intact and disrupted microsomes as well as controlling free calcium levels. Therefore work using these buffers (for example van de Werve 1989) cannot be accurately interpreted and the conclusion of van de Werve in this paper that physiological levels of calcium inhibit T1 activity in microsomes and in permeabilized hepatocytes is wrong as he did not appreciate the effect of the EGTA. However EDTA appears to have little effect on microsomal glucose-6-phosphatase activity (Mithieux *et al* 1990).

There is significant and variable binding of calcium by rat liver microsomes (Sampson & Karler 1963 and Sanui & Pace 1967). As it is difficult to control levels of free calcium in glucose-6-phosphatase assays without the buffers interfering with the assays (Mithieux *et al* 1990), workers who have shown inhibition of microsomal

glucose-6-phosphatase activity with calcium (Beaufay & De Duve 1954 and Nordlie & Arion 1965) have often used so much calcium (>10mM) that microsomal aggregation will have occurred (Kamath and Narayan 1972 and Waddell *et al* 1990).

Waddell *et al* showed that calcium activates both glucose-6-phosphatase and pyrophosphatase activity in intact microsomes (Waddell *et al* 1990), but it appeared then that from rapid filtration transport assays (using normal microsomes) that the major effect was on pyrophosphate transport and the effect on T1 activity was taken to be secondary to increased phosphate removal from the lumen of the microsomes by T2. Waddell's data appears initially to contradict the data presented above, however Waddell used "normal" microsomes and showed no effect with ≤ 0.5mM added calcium, in contrast to the "low metal ion" microsomes above which showed an effect on glucose-6-phosphate transport with 50μM added calcium in the presence of ≥ 1mM sodium. At "physiological" calcium and glucose-6-phosphate levels, calcium has been shown to have no effect on glucose-6-phosphatase activity in intact hepatic microsomes (Fulceri *et al* 1991) which again were not prepared in low calcium conditions. However glycogenolysis in hepatocytes is increased when the concentrations of free intracellular calcium are raised (Cohen 1983) in response to extracellular (eg hormonal) stimuli.

The relationship between glucose-6-phosphatase and calcium is even more complex as it has been shown that glucose-6-phosphatase activity alters free intracellular calcium concentrations (Benedetti *et al* 1985); and that in the presence of high intracellular glucose-6-phosphate concentrations calcium uptake by microsomes is increased (Benedetti *et al* 1985). Further, this pool of intra-microsomal calcium is mobilised in response to increased inositol-1,4,5-triphosphate levels (Benedetti *et al* 1987). Inositol-1,4,5-triphosphate is a postulated second messenger in hepatocyte responses to external stimuli such as hormones like insulin (Häring 1991). Similar

effects of glucose-6-phosphate on endoplasmic reticulum calcium uptake in rat kidney microsomes have been shown (Benedetti *et al* 1989).

The role of glucose-6-phosphatase and calcium in insulin release from the pancreas are discussed in Chapter 3. Three of the cases reported above (Chapter 3) had abnormalities of insulin metabolism as well as abnormalities of glucose-6-phosphatase (Cases 6,7 & 8) and all three of these cases had defects of microsomal membrane transport: further support for the role of the proteins of the glucose-6-phosphatase system in insulin release. Insulin secretion by beta cells in the pancreas is modulated by intracellular calcium levels changing in response to fluctuations in blood glucose (Colca *et al* 1983 a & b, Rorsman *et al* 1984, Hellman 1985, Morgan *et al* 1987 and Nilsson *et al* 1987). No other hormonal abnormalities were measured in any of the above cases.

In type 1b GSD (deficiency of the microsomal glucose-6-phosphate transport protein), where the neutrophils have abnormal function, defective intra-neutrophil calcium mobilisation has been demonstrated in response to certain external stimuli again suggesting that T1 may be an important controller of intracellular calcium mobilisation.

It therefore seems likely that the microsomal glucose-6-phosphate transport protein requires calcium for normal activity and plays an important role in controlling free intracellular calcium concentrations. It is also possible that increases in calcium concentration can enhance the activity of the catalytic subunit and T1 (Table 5.4.5) in the presence of >1mM potassium (which will represent the physiological state where potassium is the predominant intracellular cation).

The requirement of calcium for the stability of glucose-6-phosphate transport and the glucose-6-phosphatase catalytic subunit suggests that both are closely associated with the 21kDa stabilising protein described by Burchell & Waddell (1987b).

It did not prove possible to assess the sodium/calcium dependence of the microsomal phosphate/pyrophosphate transport protein (T2) due to the insolubility of potassium pyrophosphate when added to the "low sodium" microsomes.
It is possible that the reason that type 1b GSD is so severe is because as well as there being defective glucose-6-phosphate transport there is an associated reduction in hepatic endoplasmic reticulum sodium transport. It is also possible to speculate that the observed phenotypic differences in patients with type 1b GSD (see discussion in Chapter 3) may be explained by the fact that in some patients the T1 protein is defective, in others the sodium co-transport is abnormal and in yet others both systems are abnormal. At present there is no evidence to support this.

5.4.3 Studies on the pharmacological manipulation of T1

The ability to pharmacologically manipulate T1 has obvious advantages. In all the variants of the type 1 GSDs an increase in T1 capacity would increase the activity of the glucose-6-phosphatase system as a whole (if the protein is present), thus increasing hepatic glucose output.
Conversely in diabetes mellitus the ability to down-regulate T1 capacity would reduce the high hepatic glucose output which is found in this condition. This theory works in practice (at least in the non-diabetic) as proven by Case 8 in Chapter 3 - however hyperinsulinaemia such as was found in case 8 pretreatment would not be advantageous, as hyperinsulinaemia has been implicated in the (macro)vascular complications of diabetes mellitus (see Reaven 1988 for review). Other secondary effects on neutrophil function and lipid and purine metabolism may also be induced which would make such drugs impractical for (long-term) use. However Case 8 was not shown to have any of these abnormalities which can be found in type 1b GSD.
The effect of the diuretic drug amiloride hydrochloride [3,5-diamino-6-chloro-*N*-(diaminomethylene) pyrazine carboxamide] was examined. There were two main reasons for studying this compound. Firstly it is a potent inhibitor of several trans-

membrane sodium transport mechanisms (Sariban-Sohraby & Benos 1986 and Grinstein & Rothstein 1986) and the data above which showed the co-transport of sodium and glucose-6-phosphate suggested that amiloride may affect glucose-6-phosphate transport. Secondly, amiloride has been shown to affect the phosphorylation state of several proteins in hepatocytes as it is a non-specific inhibitor of protein kinases (LeCam *et al* 1982 and Holland *et al* 1983) and in particular amiloride inhibits insulin-stimulated phosphorylation of proteins. While there is no evidence that T1 activity is modulated by phosphorylation or by insulin it would be a logical site for insulin to exert effects on hepatocyte glucose production. This data was published (Pears *et al* 1989) and a copy of the paper can be found in Appendix A).

The effects of adding amiloride to the colourimetric assay of microsomal glucose-6-phosphatase activity in microsomes prepared from livers of fed, starved and diabetic microsomes are shown in Table 5.4.6.

Five millimolar amiloride hydrochloride was the smallest amount of the drug which produced maximal effect on microsomal glucose-6-phosphatase activity. No effect on glucose-6-phosphatase activity in any rat liver microsomal preparations was seen with the addition of less than 0.1mM amiloride to the assay. The most obvious effect of amiloride on rat liver microsomes was to decrease the Km of intact microsomes to a value approaching that of the catalytic subunit - ie reducing the latency of the microsomes. This effect was most marked in microsomes from livers of diabetic animals where T1 capacity is most rate-limiting. The mechanism of action of amiloride is obscure, but is not due to disruption of microsomes, rather due to activation of T1.

Table 5.4.6

Results of glucose-6-phosphatase activity in microsomes prepared from livers of fed, starved and streptozotocin induced diabetic rats as described in Chapter 2. All microsomes were more than 89% intact in the absence and presence of amiloride as assessed by mannose-6-phosphatase activity.

	Intact microsomes		Disrupted microsomes	
	Km (mM)	Vmax (μM/min/mg protein)	Km (mM)	Vmax (μM/min/mg protein)
a) Fed animals				
Control	3.3±0.4	0.09±0.002	0.5±0.1	0.12±0.02
1mM amiloride	1.8±0.1 **	0.09±0.02	0.5±0.2	0.11±0.02
5mM amiloride	1.4±0.2 **	0.11±0.02	0.5±0.1	0.12±0.02
b) Starved animals				
Control	3.8±0.5	0.18±0.02	1.2±0.5	0.32±0.05
1mM amiloride	2.3±0.4	0.14±0.01	0.9±0.4	0.28±0.04
5mM amiloride	1.6±0.3 **	0.17±0.01	0.7±0.2	0.25±0.01

* $p = 0.01$; ** $p < 0.01$

Data are mean ± SEM of > 4 preparations, each preparation comprising livers from 6 rats

Table 5.4.6 continued

	Intact microsomes		Disrupted microsomes	
	Km (mM)	Vmax (μM/min/mg protein)	Km (mM)	Vmax (μM/min/mg protein)
c) Diabetic animals				
Control	5.2±0.7	0.28±0.05	1.6±0.3	0.66±0.07
1mM amiloride	2.4±0.3 **	0.22±0.01	1.2±0.2	0.50±0.07
5mM amiloride	1.2±0.2 **	0.22±0.02	0.8±0.1 *	0.43±0.04

* $p = 0.01$; ** $p < 0.01$

Data are mean ± SEM of > 4 preparations, each preparation comprising livers from 6 rats

The effect of amiloride on glucose-6-phosphate transport (and accumulation of ^{14}C inside microsomes) as assessed by the radionuclide assay is shown in Figure 5.4.4. The curves in Figure 5.4.4 show that in the presence of amiloride the capacity of the glucose-6-phosphate transport protein was increased: indicated by the more rapid initial rise in retained ^{14}C to a higher peak. There was a further rise in retained ^{14}C to peak at about 2 minutes in the presence of amiloride. This second rise (which was not seen in the control microsomes) reflected an increase in total ^{14}C trapped in the microsomes. This measured ^{14}C was likely to be a mixture of labelled glucose-6-phosphate and glucose product, suggesting inhibition of T3 by amiloride as well as activation of T1.

A small decrease in the Km of the catalytic subunit was also seen in fully disrupted microsomes in the presence of amiloride. The mechanism of this is unclear and is associated with a fall in Vmax and probably represents an inhibition of the catalytic subunit by amiloride.

Attempts at measurements of microsomal pyrophosphatase activity using the colourimetric assay were unsuccessful. A precipitate formed in the low-sodium system and the assays with amiloride. This was most likely to be phosphate salts and their presence invalidated the assay.

The therapeutic concentrations of amiloride in serum are 3-20μM (Benos 1982) and micromolar amounts are used *in vitro* to inhibit transmembrane sodium channels (see Sariban-Sohraby & Benos 1986 and Frelin *et al* 1987 for example). If amiloride was inhibiting microsomal glucose-6-phosphate-linked sodium transport then the measured effect should be an increase in the Km of intact microsomes rather than the fall which was actually measured. This suggests that amiloride is not, in fact, affecting T1 activity in the microsomal glucose-6-phosphatase system secondary to an effect on sodium co-transport.

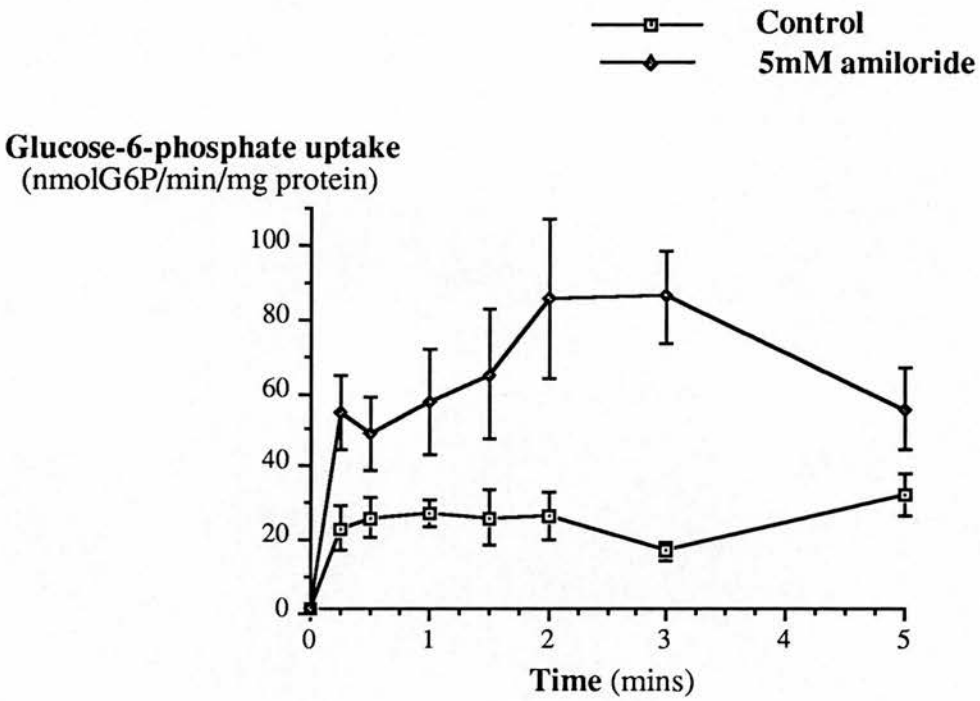


Figure 5.4.4

Results of measurements of $U^{14}C$ glucose-6-phosphate uptake by microsomes prepared from starved rat livers in the presence and absence of 5mM amiloride as described in the text. Data given of mean of >4 assays ± SEM.

This was confirmed by the addition of amiloride to colourimetric glucose-6-phosphatase assays of "low metal ion" microsomes. The results of these assays are shown in Table 5.4.7.

In the "low metal ion" assay system the addition of 5mM amiloride was sufficient to remove the rate-limitation placed on the activity of the glucose-6-phosphatase system by T1 capacity. The Km of intact microsomes in the presence of amiloride approached that of disrupted microsomes - a situation identical to amiloride's effect on "normal" microsomes (Table 5.4.6). Again amiloride is not working by disrupting microsomes - the intactness of all intact microsomes as assessed by mannose-6-phosphatase activity was greater than 85% in both "normal" and "low metal ion" assay systems and the data in Tables 5.4.6 & 5.4.7 has been corrected for intactness.

It is also unlikely that amiloride is acting by altering microsomal protein kinase activity and, therefore, the phosphorylation state of T1 as no extrinsic source of high-energy phosphate (eg ATP or GTP) was added to the system.

The simplest explanation of these data therefore is that amiloride is directly interacting with T1 and T3 rather than affecting sodium transport. It is not clear from this data if amiloride is increasing the capacity of a fixed number of T1 molecules, or increasing the number of active T1 molecules, each of which works at a fixed capacity. The mechanism of the effect on T3 is likewise unclear.

The importance of this data is the first demonstration that T1 can be acutely manipulated.

Table 5.4.7

Results of colourimetric glucose-6-phosphatase assays of "low metal ion" microsomes prepared from the livers of starved and streptozotocin-induced diabetic rats using potassium glucose-6-phosphate substrate in the presence and absence of amiloride compared to "normal" microsomes assayed with sodium glucose-6-phosphate.

Microsomal preparation	Substrate	Intact microsomes Km (mM)	Intact microsomes Vmax (μmol/min/mg)	Disrupted microsomes Km (mM)	Disrupted microsomes Vmax (μmol/min/mg)
a) Starved animals					
Normal	Na G6P	3.8±0.5	0.18±0.02	1.2±0.5	0.32±0.05
Low metal ion	K G6P	16.3±2.1	0.39±0.1	0.5±0.1	0.21±0.03
Low metal ion	K G6P+5mM amiloride	2.3±0.4	0.23±0.04	1.0±0.3	0.31±0.06
b) Diabetic animals					
Normal	Na G6P	5.2±0.7	0.28±0.05	1.6±0.3	0.66±0.07
Low metal ion	K G6P	14.4±2.4	0.45±0.08	1.1±0.2	0.4±0.03
Low metal ion	K G6P+5mM amiloride	2.0±0.3	0.23±0.03	0.8±0.1	0.4±0.02

All results are mean ± SEM of at least 4 assays

5.4.4 Discussion

Amiloride does not affect glucose tolerance in man, unlike other diuretics, and is unlikely to accumulate in hepatocytes to the concentrations required to *in vitro* affect T1 and T3 as it has been reported not to cross cell membranes (Benos 1982). However this is the first demonstration of *in vitro* pharmacological manipulation of T1. It may therefore become possible to pharmacologically manipulate T1 capacity *in vivo* thereby increasing hepatic glucose production in patients such as those in Chapter 3 above, and reducing the hyperglycaemia of diabetes mellitus.

The data in Table 5.4.6 also suggests that amiloride was inhibiting T3, the microsomal glucose transporter. The fact that the Vmax of intact microsomes from the livers of starved and diabetic rats fell in the presence of increasing concentrations of amiloride rather than rising to approach the Vmax of the catalytic subunit (when the Km was almost at the level of the Km of the catalytic subunit) suggests inhibition of the catalytic subunit in intact microsomes in the presence of amiloride by reaction product. The simplest explanation for this data and the retention of ^{14}C shown in Figure 5.4.4 is that amiloride is inhibiting T3 also resulting in an increase in glucose concentrations in the lumen of the endoplasmic reticulum. Arion *et al* (1980b) demonstrated a 52% fall in the Vmax of the glucose-6-phosphatase catalytic subunit with high glucose levels (5mM amiloride caused a 51% fall in the Vmax of the catalytic subunit) and a 53% increase in the Km of glucose-6-phosphatase activity of intact microsomes (in the presence of 5mM amiloride the Km in intact microsomes was 50% higher than in disrupted structures).

Several agents have been described which will modulate microsomal glucose-6-phosphatase activity (see Nordlie & Sukalski 1985 for a review). Most of these effects are inhibitory and the mechanism of the effect is not clear except for pyridoxal phosphate, phlorizin and DIDS which (amongst many protein interactions) are known to be inhibitors of T1 (Gold & Widnell 1976, Arion *et al*

1978, 1980 a & b, Zoccoli *et al* 1980 and 1982 and Waddell & Burchell 1987). However polyamines and histone 2A have been shown to have stimulatory effects on glucose-6-phosphatase activity in intact microsomes (Benedetto *et al* 1979, Nordlie *et al* 1979a and Vergnes *et al* 1981). These latter effects have recently been shown to be due to disruption of the integrity of the microsomal membrane and removal of the rate-limitation imposed by the transport proteins rather than to actions on parts of the glucose-6-phosphatase system. This is the basis for the incorporation of histone 2A in the glucose-6-phosphatase assays used in this work to assay the activity of the catalytic subunit (Blair & Burchell 1988).

The effect of amiloride as a specific activator of T1 is the first description of a compound definitely activating T1 rather than poisoning it (with a number of other transport systems) as DIDS, pyridoxal phosphate and phlorizin do.

Since the effect of amiloride was demonstrated on microsomal glucose-6-phosphate transport capacity, the effect of other agents has been examined. This later data however does not present clear effects of most of these compounds on glucose-6-phosphatase activity and more work with them will also be needed to establish the precise mechanism by which these compounds affect glucose-6-phosphatase. In my opinion in order to clearly demonstrate T1 activity both the kinetic data of the colourimetric glucose-6-phosphatase assay and the direct measure of trace radioactive isotope uptake by microsomes is required. This data from these two assay techniques will also be useful for characterising the inter-reactions of the protein components of the glucose-6-phosphatase complex.

The following data summarises the effects of some compounds on the glucose-6-phosphatase system which are suggested as being T1 effects and which have been measured since my work with amiloride. Unfortunately most of this work does not contain data using the direct radioisotope measure of T1 capacity.

The sulphated derivatives of dehydroepiandrosterone (DHEA-S) and oestrone (E-S) have been demonstrated to have *in vitro* inhibitory effects on hepatic microsomal T1

and, to a lesser extent, T2 capacity (Scott *et al* 1991). The unsulphated forms had no such effects, but DHEA when fed to rats is well recognised to be anti-hyperglycaemic in animal models of diabetes (Coleman *et al* 1982 & 1984). It is possible that the mechanism of action for this is inhibition of T1 $\pm$ T2 by DHEA-S. Of further interest is that the inhibitory effect of DHEA-S and E-S was reversible *in vitro* by preincubation with 10mg/ml BSA (as was the microsomal T1 inhibitor found in case 8 in Chapter 3).

Pentamidine (at pharmacological concentrations) appears to affect microsomal T1 activity in the same way as amiloride, (Scott & Burchell 1991a). The prostaglandins D_2, E_2 and $F_{2\alpha}$ were shown to produce a very slight decrease in the Km of microsomal glucose-6-phosphatase activity in intact microsomes (Orme *et al* 1990) and the tricyclic antidepressant imipramine and verapamil (a calcium channel antagonist) cause activation of glucose-6-phosphatase in intact microsomes (Scott & Burchell 1991b). Similarly alamethicin (a pore forming antibiotic) activates glucose-6-phosphatase in intact microsomes without apparently disrupting them (Voice & Burchell 1991).

The *in vitro* effect of glycogen on the glucose-6-phosphatase system was a marked inhibition of glucose-6-phosphatase activity in intact microsomes from livers of starved rats (Grant & Burchell 1990a). This inhibition (a marked fall in Vmax with a lesser fall in Km) is different to the pattern I demonstrated in the "low metal ion" system presented above where inhibition of T1 resulted in a very high Km and a smaller increase in Vmax. Differing methods of inhibition (eg competitive *vs* non-competitive) will result in differing patterns of kinetic constants: it is not yet known what the inhibitory mechanisms of glycogen excess and sodium lack are. Glycogen blocks the pores of the nitrocellulose membrane, making direct T1 capacity measurement difficult.

The effects of ATP and GTP were assessed however by the direct radionuclide T1 assay, and both produced an increase in ^{14}C retained by intact microsomes. This

peak in retained ^{14}C was after about 2 minutes incubation (the same time as the second peak seen in microsomes incubated with amiloride - Figure 5.4.4) and I feel more properly represents inhibition of T3 as indeed was postulated by the authors (Grant & Burchell 1990b).

Amiloride (and pentamidine) added to intact hepatocytes (Grant *et al* 1991) showed an apparent inhibition of glucose-6-phosphatase catalytic subunit activity. However closer examination of this data suggests that there was in fact an activating effect of amiloride on T1 capacity in these hepatocytes, but that this was masked by a marked inhibitory effect on the enzyme.

The effect of pentamidine is interesting as it is well recognised to cause hyperglycaemia *in vivo* (occasionally due to pancreatic beta cell toxicity - Bouchard *et al* 1982 and Zhon & Ipp 1989) which may be permanent or transient and followed by prolonged hypoglycaemia (Kapusnik & Mills 1986). The mechanisms of the actions of pentamidine on glucose tolerance in man are unclear. Based on the data above it would be interesting to study glucose-6-phosphatase activity in patients with disturbed glucose tolerance induced by pentamidine. Similarly the action of diazoxide in reducing pancreatic insulin secretion is not clear and may possibly be via an effect on islet cell glucose-6-phosphatase. In patients taking pentamidine, diazoxide or any drugs affecting glucose metabolism and patients with Addison's disease (adrenal hypofunction associated with low blood glucose levels) and Cushing's syndrome (glucocorticoid excess) the study of microsomal glucose-6-phosphatase activity is difficult as liver biopsy is still required and impossible to do routinely or repeatedly (for example before and after treatment). The ability to perform such measures would give important data on the physiology of glucose-6-phosphatase and glucose homeostasis and was, therefore further impetus to the attempts to develop an assay of glucose-6-phosphatase activity in circulating neutrophils described in Chapter 4 above.

SUMMARY OF DISCUSSION

The capacity of the hepatic microsomal glucose-6-phosphate transport protein, T1, is rate-limiting to the activity of the whole glucose-6-phosphatase system and, therefore, to hepatic glucose production. Little was previously known of this key protein in glucose homeostasis. I was interested in it for two reasons: firstly because of its key role understanding its structure and function is important to understanding hepatic glucose production and secondly understanding the protein and its mechanism of action will be important in trying to pharmacologically manipulate the capacity of T1 to alter hepatic glucose production in, for example, the eight cases presented in Chapter 3.

A rat hepatic microsomal protein of molecular weight approximately 66kDa (as assessed from a silver stained 10% SDS gel) was isolated by affinity chromatography using Sepharose-bound DIDS as ligand. DIDS is known to bind to glucose-6-phosphate binding sites and is a specific inhibitor of the hepatic microsomal glucose-6-phosphate transport protein, T1. An antibody to the isolated protein was raised in a sheep and the IgG fraction of serum was used to try and identify the protein. The antibody had no obvious effect on microsomal glucose-6-phosphatase activity in intact preparations from starved and diabetic rats. This does not mean that the IgG antibody is not recognising the glucose-6-phosphate transport protein as the polyclonal antibodies to the glucose-6-phosphatase catalytic subunit protein and T2 do not affect the function of their target proteins. Immunoblot analysis of microsomes separated by using the isolated IgG as primary antibody identified several proteins: one at a molecular weight of approximately 35kDa and another at about 50kDa were the most strongly recognised. There was more of both of these proteins in microsomes from diabetic animals than starved and fed animals. The 35kDa protein has approximately the same molecular weight as the glucose-6-phosphatase catalytic subunit and there is more catalytic subunit protein in

microsomes from diabetic rat livers, suggesting that the antibody has possibly recognised the glucose-6-phosphatase catalytic subunit. However, perhaps of more relevance is the very recent identification of a microsomal protein of approximate molecular weight 35kDa which strongly binds radiolabelled glucose-6-phosphate (Ann Burchell, personal communication). This suggests that the 35kDa protein identified by the antibody I prepared may turn out to be T1. It is possible that the 35kDa protein was present in the original fractions from the DIDS/Sepharose affinity chromatography but did not (like the glucose-6-phosphatase catalytic subunit protein) show well on a silver stain.

There is as yet no clear evidence that the 66kDa, 50kDa or 35kDa proteins are in any way related to the microsomal glucose-6-phosphatase system.

Much further work on these proteins is necessary to definitely identify them. The next major step is to reconstitute the protein(s) into liposomes and try and identify glucose-6-phosphate uptake. This reconstitution and demonstration of glucose-6-phosphate uptake would rely on the clarification of the biochemical mechanism of glucose-6-phosphate transport.

In parallel with this work on isolating a protein I was able to show, using a novel "low metal ion" method of producing microsomes and assay system, that hepatic microsomal glucose-6-phosphate transport is associated with sodium co-transport across the microsomal membrane. This possibility had been examined before following the assumption that glucose-6-phosphate exists as an anion, but the earlier investigators had added more sodium to microsomes and buffers already containing sodium and shown no effect. Only by removing "all" sodium (by using potassium salts) was I able to demonstrate the requirement for sodium co-transport both by inference from the kinetics of the colourimetric glucose-6-phosphatase assay and directly by measuring increased sodium uptake by "low metal ion" microsomes in the presence of glucose-6-phosphate. This co-transport of a cation will preserve electrostatic neutrality across the endoplasmic reticulum membrane and as sodium is

not the predominant intracellular cation it would appear the logical choice to control glucose-6-phosphate transport. The stoichiometry of glucose-6-phosphate and sodium transport was not investigated.

As well as showing this co-transport I showed that the transport of either one or both of glucose-6-phosphate and sodium requires a divalent metal cation (at $< \mu M$ concentrations), probably calcium (although a number of other cations were shown to have an effect). This raised the possibility that T1 requires the calcium-binding stabilising protein identified by Burchell & Waddell for normal function.

The relationship between calcium and the microsomal glucose-6-phosphatase system has been the source of much interest over many years, since the observations by Nordlie & Johns in 1967 and 1968 that metal-binding agents inhibited catalytic subunit activity. Much of the work has been complicated by the use of EGTA and NTA as buffers which themselves have recently been shown to affect glucose-6-phosphatase activity and by the fact that there is significant and variable non-specific binding of calcium to microsomes, such that at added calcium concentrations approaching 10mM microsomes clump together and precipitate out. The data above is the first on "low metal ion" microsomes. Apart from the requirement of the stabilising protein for calcium it seems clear that glucose-6-phosphatase (including, from my data,T1) have a role to play in mobilising endoplasmic reticulum calcium stores in response to extracellular stimuli such as hormones in hepatocytes and pancreatic islets.

Following from this work on sodium I was then able to demonstrate that the capacity of T1 could be pharmacologically manipulated *in vitro* by the sodium channel inhibitor amiloride. Amiloride also appeared to inhibit the microsomal glucose transporter T3/Glut 7. However the effect of amiloride on T1 transport was shown to be not due to an effect on microsomal sodium transport. Since the effects of amiloride was demonstrated, there have been attempts to show that other agents effect T1 capacity, but this data has not always been convincing. The observed

effects of pentamidine on glucose metabolism in man, however, would be consistent with its demonstrated activation of T1 in the test tube. However, without using a liver biopsy, it is still not possible to reliably study changes in glucose-6-phosphatase activity in man (see Chapter 4).

Case 8 in Chapter 3 had already indicated that T1 could be inhibited and that the effect of this inhibition may be beneficial to the increased hepatic glucose output seen in diabetes mellitus.

However it is important to realise that T1 capacity can be manipulated as this can be used to target agents to alter hepatic glucose output.

CHAPTER 6

CONCLUDING REMARKS

In this thesis I have presented the data from my studies over 2 years on the glucose-6-phosphatase system in both man and the rat. These studies have improved the understanding of both the clinical relevance of glucose-6-phosphatase in man and of the biochemistry of the enzyme system with particular emphasis on the microsomal glucose-6-phosphate transport protein, T1.

The presentation of the ten adult cases with newly diagnosed glycogen metabolising enzyme abnormalities in Chapter 3 and Appendix C is the most detailed description to date of such individuals. Such patients are undoubtedly more common than has previously been recognised. With the publication of the descriptions of these individuals (Pears *et al* 1992) and the ability to investigate such patients, more cases will be diagnosed, further helping to increase our understanding of the glycogenolytic enzymes. Two of the cases described (numbers 7 and 8) are of previously unrecognised abnormalities of glucose-6-phosphatase activity. Case 7 has similar low levels of activity of all assayed components of the glucose-6-phosphatase system to some premature infants (these data were subsequently published by Burchell *et al* 1990) implying that case 7 may have had abnormal development of the hepatic glucose-6-phosphatase system *in utero*. The reason for this interruption in development is not clear from the history of her mother's pregnancy. The patient was born 3 weeks prematurely, but so are many babies who go on to have no problem with glycogen metabolism - possibly the development of glucose-6-phosphatase in case 7 could not be or was not resumed after the insult which caused the initial delay. Despite the data on the ontogeny of the human glucose-6-phosphatase presented by Burchell *et al* (1990) it is not clear at which

point her development was affected. An alternative explanation for the low assayed glucose-6-phosphatase activity in her liver sample is a defect in the regulation of turnover of the proteins of the glucose-6-phosphatase system. Nothing is yet known of the turnover times of the glucose-6-phosphatase system proteins nor of the site and regulation of expression of the genes involved. As more cases are recognised and the regulation of the genes involved becomes apparent the nature of the underlying defects will become clearer. Similarly case 8 was, and still is, unique in an adult: not in his presentation with reactive hypoglycaemia but in the diagnosis of the inhibitor of the microsomal glucose-6-phosphate transport protein, T1, in his liver (and presumably pancreas). This abnormality (which, interestingly did not cause glycogen accumulation in his liver) has been identified in infants and has been called pseudo-type 1b GSD by Ann Burchell (Burchell & Gibb 1991). There are a recognised sub-group of patients with reactive, post-prandial hypoglycaemia who, like case 8, have marked hyperinsulinism, and a further sub-group of these in whom somatostatin abolishes the abnormality (Baschieri *et al* 1989). It seems highly likely that at least some of these people will have an identical underlying defect to case 8, and we would be very interested to study liver biopsy samples from them. This case is different to the other seven in that he does not appear to have a defective protein component of the glucose-6-phosphatase system. As he was well until his late twenties it is also possible that his abnormality was acquired. It has not been possible to identify the nature of the T1 inhibitor in this man or in any of the infants shown to have an inhibitor of T1 (Burchell *et al* 1990 and Burchell & Gibb 1991). Many of these cases point to the role of glucose-6-phosphatase in other tissues, especially pancreatic islets, where the proteins of the glucose-6-phosphatase system may play an important role in the regulation of insulin secretion (and possibly glucagon or somatostatin - see the discussion in Chapter 3).

The symptoms described by these patients were vague and often difficult to ascribe to a single abnormality such as hypoglycaemia worsened by fasting and exercise. In

our experience, it has often been very difficult to biochemically prove hypoglycaemia. It is likely that patients such as these have not previously been recognised as having an organic disease as the diagnosis was (understandably) not considered or the appropriate investigations were not performed.

The investigations of the patients presented here indicate a clear and logical method for investigating patients with symptoms which may be caused by hypoglycaemia. A high index of suspicion must exist that the vague symptoms may be organic in origin and referral to a clinical psychologist should be made after all organic disease has been excluded (not as in the mother in Appendix C). A temporal relationship of onset of symptoms to fasting followed by exercise and/or their improvement by ingestion of food should especially be sought. Although liver enzyme defects such as these may be commoner than previously recognised, there are still other, possibly commoner causes of hypoglycaemia, such as insulinoma. A 48 hour fast with blood samples every 12 hours (and at the time of symptoms) for glucose and insulin levels will exclude the vast majority of insulinomas. The patients with partial glucose-6-phosphatase (or phosphorylase kinase) abnormalities may not become hypoglycaemic after a 48 hour fast and exercise: the investigations should proceed in the absence of proven hypoglycaemia. A standard 75g oral glucose tolerance test should be performed (extended if reactive hypoglycaemia such as in Case 8 is suggested) to examine for impaired glucose tolerance or diabetes as demonstrated by cases 6, 7 and 8 above. A glucagon test after an overnight fast as described in Chapter 2 is the screening test which we propose and use for patients suspected of having hepatic glycogenolytic enzyme defects. A rise in blood glucose of $>$4mmol/l is taken as normal, and less than this is suspicious. This criterion of normality is something we have arrived at after the description of Fernandes *et al* (1969) and is less strict than that applied routinely to children (Dunger & Leonard 1982). The sensitivity and specificity of the glucagon test as we use it is unknown. We have not performed liver biopsies on patients who do not meet the criterion for

a normal response to glucagon which we have applied and nor have we performed glucagon tests on subjects undergoing liver biopsy for other reasons. The reproducibility of the glucagon test within the same individual on different occasions, or the importance of the feeding state of the subject when the glucagon test is performed is not known. It is possible, for example, that for maximum responsiveness to injected glucagon there is a threshold time that a subject must fast before the test is carried out. I plan to answer some of these questions regarding glucagon test reproducibility in healthy volunteers in the near future. However until a less invasive way of studying glucose-6-phosphatase becomes possible, most of these problems will remain unsolved.

Three of the cases described in Chapter 3 demonstrated biochemical hypoglycaemia after glucagon administration. Interestingly so did the majority of a pooled series of type 1b GSD patients described by Ambruso *et al* (1985). Unfortunately there is no data on insulin secretion in response to a glucose load in type 1b GSD patients, it is possible that some, if not all, demonstrate hyperinsulinism to a rise in blood sugar levels.

If a patient's symptoms are severe enough in the face of a blunted glucose response to glucagon then a liver biopsy should be considered and discussed with the patient. Some patients take the view that knowing the exact hepatic defect (if it exists) is not going to alter their management and choose to have a trial of dietary therapy. This decision is entirely understandable in view of the invasive nature of a liver biopsy, but is frustrating for those trying to identify enzyme defects and collect as much data as possible on these subjects (including blood for DNA analysis when the specific gene probes for the proteins of the glucose-6-phosphatase system become available).

Before the liver biopsy, arrangements must be made with a pathologist (for electron microscopy) and a laboratory who regularly and reliably assay such samples for

glucose-6-phosphatase and other enzyme activities as necessary, for rapid handling of the liver sample.

The mainstay of treatment for these subjects has been dietary manipulation - a diet during the day high in refined carbohydrate with uncooked corn starch supplementation as has routinely been used for older children with type 1 GSD patients for a number of years now. This therapy was sufficient alone for most of the cases presented above. Case 8 was again unusual in that he required drug treatment which was targeted at reducing his hyperinsulinism: diazoxide is a standard therapy for pancreatic nesidioblastosis in children (hypoglycaemia due to excessive insulin secretion from diffuse hyperplasia of pancreatic islets rather than from a discrete insulin-secreting tumour).

The beneficial effect of oestrogen hormone replacement therapy on the symptoms of hypoglycaemia in three women after the menopause has stimulated *in vitro* work on the effects of steroid hormones on glucose-6-phosphatase activity, and some effects have been shown (Scott *et al* 1991). This effect of sex hormones on glucose-6-phosphatase is consistent with observations by clinicians of an improvement in symptoms of hypoglycaemia (Greene *et al* 1981) and increased hepatic glucose output in children with type 1 GSD around the time of puberty (Powell *et al* 1981, Kalhan *et al* 1982 and Tsalikian *et al* 1984).

In all the cases described it would have been useful to have been able to make repeated assay measurements of glucose-6-phosphatase activity in, for example, the same individual on a number of days; in different feeding states; and especially while on treatment with either diazoxide or oestrogen replacement. Data collected this way would be invaluable not least to give an idea about the day-to-day reproducibility of the assay data in the same individual. The effects of treatment measured by changes in glucose-6-phosphatase activity would have given useful information on the *in vivo* regulation of glucose-6-phosphatase.

The natural history of these patients is unclear (apart from cases 1 and 6 above), but they are, wherever possible, being followed as out-patients with special reference to the development of hepatic tumours (and possibly other tumours) and the other recognised complications of type 1 GSD.

As well as being new diagnostic entities these patients provide important information about the glucose-6-phosphatase system and inter-relationship of the proteins in the system *in vivo*.

The use of a tissue more accessible than liver for studying patients such as these, patients with other conditions associated with abnormal glucose tolerance and patients using treatments which interfere with glucose homeostasis is unfortunately still not possible.

Normal adult human intestinal mucosa was proven for the first time in this thesis to contain specific glucose-6-phosphatase (using immunoblot analysis with antibody monospecific for the glucose-6-phosphatase catalytic subunit - Chapter 4). However the problem of proteolysis of the samples even in the presence of several combinations of protease inhibitors has yet to be resolved to allow reliable assay measurements of the low levels of specific glucose-6-phosphatase activity. It is therefore clear that intestinal mucosa is not at present a suitable tissue on which to diagnose abnormalities of glucose-6-phosphatase *in vivo*.

A blood fraction would be the most convenient tissue of all for the patient and physician on which to study glucose-6-phosphatase activity. The glucose-6-phosphatase enzyme was proven above to be present in neutrophils separated from normal adult serum by immunoblot analysis (Chapter 4). However the problem of proteolytic damage again occurred when trying to study the enzyme in these cells. It seems likely that the activity of all phosphatases (including glucose-6-phosphatase) is so low (or sensitive to proteolysis) that the colourimetric phosphatase assay employed in this work is not sensitive enough. For assay purposes a method of amplification of the colour produced by the released inorganic phosphate would be

required to allow glucose-6-phosphatase activity in neutrophils to be demonstrated. The use of higher concentrations of substrate and/or longer assays did not solve the problem - a more sensitive way of detecting free phosphate is needed. This work is important and, once these problems have been overcome, will make the *in vivo* study of this important part of glucose homeostasis much easier.

The presence of glucose-6-phosphatase activity in intestinal mucosa and neutrophils explains some of the clinical abnormalities seen in patients with type 1 GSD (see Hers *et al* 1989 and Moses 1990 for reviews). Many type 1 GSD patients have diarrhoea, especially after a glucose load, however malabsorption is not seen clinically (Fine *et al* 1969 & Milla *et al* 1978). There are recognised radiographic findings identical to those seen in colitis in some patients with type 1 GSD even in the absence of gastrointestinal symptoms (Fellows *et al* 1975). More recently histologically proven Crohn's-like colitis has been described in a a number of type 1b GSD patients (for example Sanderson *et al* 1991). The pathogenesis of this colitis is unclear as is the physiological role of glucose-6-phosphatase in intestinal mucosa. There is evidence from rat colonocytes that glucose-6-phosphatase activity is central to providing energy for the normal metabolism of these cells which differentiate very rapidly (Ardawi & Newsholme 1985 for example). Another possible role of the enzyme would be in the transport of absorbed glucose across the intestinal mucosa.

Neutrophil dysfunction is a characteristic finding in type 1b GSD (Anderson *et al* 1981) and recently defective calcium mobilisation in neutrophils separated from patients with type 1b GSD in response to some stimuli has been shown (Kilpatrick *et al* 1990). Calcium mobilisation is important for producing the respiratory burst in neutrophils (Heyworth & Segal 1986) all of which is consistent with the observations of decreased neutrophil function in type 1b GSD. The role of glucose-6-phosphatase in the pancreatic islets was postulated by Waddell & Burchell (1989) to be related to control of insulin secretion secondary to an effect on free

intracellular calcium levels. This is consistent with other data on the relationship *in vivo* between glucose-6-phosphatase activity and cytosolic free calcium levels suggesting that glucose-6-phosphatase activity somehow controls calcium levels in response to extracellular (hormonal) stimuli rather than *vice versa* (Benedetti *et al* 1985, 1986 & 1988, Waddell & Burchell 1988 & Fulceri 1990). A lot of the literature presenting so-called effects of calcium on glucose-6-phosphatase activity is erroneous in that some of the effects described were due to the buffer systems used (mainly EGTA, but NTA also has a significant effect) affecting the microsomal glucose-6-phosphatase activity (see Waddell *et al* 1990 and Mithieux *et al* 1990). Before the work presented in this thesis the only proven biochemical interaction between calcium and the glucose-6-phosphatase system was the requirement of the 21kDa stabilising protein for μM concentrations of calcium to allow normal glucose-6-phosphatase catalytic subunit activity (Burchell & Waddell 1990b). The data in Chapter 5 here indicates a role for calcium (also at μM concentrations) in the normal transport of glucose-6-phosphate across the microsomal membrane. It is not yet clear if the glucose-6-phosphate transport protein, T1, is closely associated with the stabilising protein or whether T1 itself requires calcium for normal function. The model of the glucose-6-phosphatase system may in fact be best represented with the stabilising protein at the centre (see Figure 6.1). Perhaps the stabilising protein also acts as a regulating protein - in the presence of high free intracellular calcium concentrations secondary to hormone receptor stimulation more calcium binds to the glucose-6-phosphatase stabilising protein thus increasing intracellular glucose-6-phosphatase activity (both T1 capacity and catalytic subunit activity), which increases glucose production and also reduces free calcium levels again. This theory is consistent with the observation that glycogenolysis is increased in hepatocytes when the levels of free cytosolic calcium are raised (Cohen 1983) and the work of Benedetti showing that calcium uptake by microsomes is increased in the presence of high intracellular glucose-6-phosphate concentrations (Benedetti *et al* 1985).

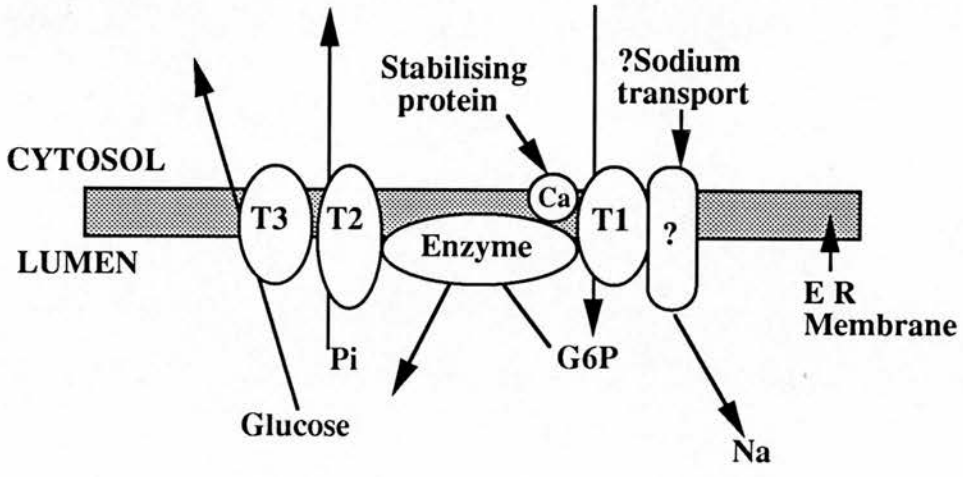


Figure 6.1

Representation of the hepatic microsomal glucose-6-phosphatase system as it was visualised at the end of the work in this thesis.

Subsequent to my data the effect of supra-physiological levels of calcium on the capacity of the phosphate/pyrophosphate and glucose-6-phosphate transport proteins added to normal microsomes was described (Waddell *et al* 1990). The capacity of the T2 transport protein under those circumstances increased more than that of T1. Unfortunately I was unable to study T2 activity in the "low metal ion" system which I developed due to the insolubility of the non-sodium pyrophosphate salts. However I did not observe an effect which could be taken as phosphate inhibition of microsomal glucose-6-phosphatase activity in the low metal ion system suggesting that T2 was functioning to some extent under those conditions.

The rate-limitation of the capacity of the glucose-6-phosphate transport protein T1 to glucose-6-phosphatase activity in intact microsomes is well recognised (Arion *et al* 1980b) and therefore understanding its structure and function is important in the physiology of health and disease.

For the first time I have demonstrated (in Chapter 5) the biochemical mechanism for microsomal glucose-6-phosphate transport - glucose-6-phosphate co-transports with sodium and this transport is dependent upon calcium. It was not established here whether glucose-6-phosphate and sodium co-transport by the same or by closely associated proteins and the stoichiometry of the co-transport has still to be established; 1x glucose-6-phosphate : 2x sodium would be the most likely.

The 66kDa protein which I isolated from microsomes from diabetic rat livers (Chapter 5) has yet to be properly identified. There is as yet no firm evidence that it is even part of the glucose-6-phosphatase system. Very recently work in Ann Burchell's laboratory using radiolabelled glucose has suggested that T1 may in fact be a protein of molecular weight 35kDa (the same size as a protein recognised by the polyclonal antibody raised to fractions from affinity chromatography which contain the isolated protein). The absolute proof as to whether a protein is T1 is its reconstitution into liposomes and the subsequent demonstration of glucose-6-phosphate transport. Work such as this could easily occupy several years'

continuous effort - the isolated protein may well not be in the tertiary structure necessary to allow glucose-6-phosphate transport, and if the sodium co-transport occurs via a separate protein the two proteins would have to be reconstituted together, both in working structures, to allow the demonstration of glucose-6-phosphate transport. This work has not yet been started.

The 66kDa isolated protein was used to raise an antibody which, on immunblot analysis, recognised 2 main rat hepatic microsomal proteins one of apparent molecular weight 35kDa and another of 50kDa. There was more of both of these proteins in microsomes from diabetic compared to starved rat livers which could support the 35kDa protein being glucose-6-phosphatase catalytic subunit. However kinetic analysis has suggested that there is no increase in T1 capacity in microsomes from the livers of diabetic animals. The fractions from the DIDS/Sepharose affinity chromatography may have contained more than one protein, and only the one at 66kDa took up the silver stain. Looking at Figure 5.3.1 in retrospect and with the recent knowledge of the radiolabelled glucose-6-phosphate data, there may be a protein band at about 30kDa. However this band was definitely not present on stained gels of other fractions which contained the 66kDa protein although the protein may have been present in the fractions.

A useful indicator to the identity of the isolated protein would be to immunoblot microsomes prepared from the liver of a patient with type 1b GSD with IgG raised to the original isolated protein. This has not yet been possible.

The IgG to the isolated protein was added to a small number of microsomal glucose-6-phosphatase assays and had no effect. This does not mean that the antibody is not binding to T1. The antibody was also used as a ligand on an affinity column and isolated a single peptide which on a silver stained 10% polyacrylamide SDS gel had an apparent molecular weight of 66kDa. No other proteins were stained.

The original protein I isolated is larger than the protein identified by Zoccoli *et al* (1982) using radiolabelled DIDS which was 54kDa and was subsequently shown to be a hepatocyte cell membrane transport protein (Ananthanorayanan 1988). My microsomal preparations contained little cell membrane contamination (as shown by the presence of little precipitate when they were incubated with concanavalin A), nor did I wash the microsomes which had previously been shown to increase the binding of DIDS to microsomes (Countaway & Arion 1986). Although DIDS does bind to many microsomal proteins (Countaway & Arion 1986) and to anion transporters (Cabantchik & Rothstein 1972) the methods I used appeared initially to be successful at isolating only one protein.

One of my aims was to demonstrate that T1 capacity could be pharmacologically regulated. The ability to do this *in vivo* could have a number of important applications. The cases with hypoglycaemia presented in Chapter 3 (and possibly those in Appendix C) could have all benefited from the increased hepatic glucose output which would occur if T1 capacity was increased, removing its rate-limitation to glucose-6-phosphatase activity, and allowing even low levels of glucose-6-phosphatase catalytic subunit to produce increased amounts of glucose. Secondly in patients with diabetes mellitus, where hepatic glucose output is increased, if the rate limitation imposed by T1 on glucose-6-phosphatase activity could be increased (ie induction of partial pseudo type 1b GSD) then hepatic glucose output could be decreased. However decreasing T1 capacity could in theory cause all the other metabolic problems seen in type 1b GSD patients (see Hers *et al* 1989 and Moses 1990), or at very least the exaggerated insulin and blunted glucagon responses to fluctuations in blood glucose demonstrated in case 8 in Chapter 3. These effects of such an agent, if they occurred, could make its use unacceptable. It is possible in time that glucose-6-phosphatase in different tissues represent isoenzymes and that the proteins of the systems in these isomorphs have differing specificities for pharmacological agents, thus these agents could be targeted to each tissue. At

present there is no evidence of isoenzymes of glucose-6-phosphatase, the smaller molecular weight glucose-6-phosphatase catalytic subunit seen on immunoblots of gut mucosa and neutrophils represents proteolytic damage.

Before I started my work no agents had been shown to definitely manipulate T1 activity. A number of translocase poisons (eg phlorizin and DIDS) had been demonstrated and some agents which removed or decreased the rate-limitation imposed by T1 (eg histone 2A and detergents). These latter effects were subsequently shown to be due to disruption of the integrity of the microsomal membrane. I studied the diuretic amiloride for two reasons. Firstly it is a potent inhibitor of transmembrane sodium channels and, after the data demonstrating co-transport of sodium with glucose-6-phosphate, it seemed a logical agent to use. Secondly amiloride affects the phosphorylation states of several insulin-sensitive proteins within hepatocytes (LeCam *et al* 1982 and Holland *et al* 1983). While T1 has not been shown to be under the control of insulin or be regulated by phosphorylation, it would be a logical site of insulin action in hepatocytes to decrease hepatic glucose production. The effects of high concentrations of amiloride were dramatic: the activation of T1 was profound (as demonstrated by the colourimetric assay and the specific assay of T1 capacity) and there is evidence that amiloride also inhibited T3, the glucose transport protein of the glucose-6-phosphatase complex. However this is the opposite effect on T1 than was expected if amiloride was inhibiting the sodium co-transport mechanism and the concentration of amiloride required to produce maximal effect (5mM) is very much higher than has been needed before to inhibit transmembrane sodium channels. Amiloride was subsequently incubated with intact freshly isolated hepatocytes and a profound effect on glucose-6-phosphatase activity was seen which may have masked any effect on glucose-6-phosphate transport (Grant *et al* 1991). Amiloride does not affect glucose metabolism *in vivo* in man, presumably because the concentration of

the drug does not get high enough inside hepatocytes (if amiloride enters these cells) or because T1 is not insulin regulated.

Since this work on amiloride demonstrated clearly that T1 could be acutely regulated workers in Ann Burchell's laboratory have shown effects of a number of other compounds on rat liver microsomal glucose-6-phosphatase which they claim to be a T1 effect (see Chapter 5).

Of these agents the one which is known to effect glucose metabolism in man is pentamidine and it would be interesting to see if *in vivo* measures of T1 capacity confirmed the activation described *in vitro* (Scott & Burchell 1991a) to explain the action of this drug on glucose metabolism. Unfortunately this is also unrealistic until a method of assaying glucose-6-phosphatase activity in, for example, circulating neutrophils becomes available.

The model of the glucose-6-phosphatase enzyme system as discussed in the introduction to this thesis has, during the time of my work, moved more and more towards the multicomponent model (see Burchell 1992 for a review). The sequence of the rat microsomal glucose-6-phosphatase catalytic subunit has been published. The T3 (or Glut 7) transport protein has been sequenced, cloned and expressed (Waddell *et al* 1992). A recent paper has also challenged the concept of a single T2 transporter by examining the difference between the observed and predicted inhibition of glucose-6-phosphatase activity by accumulated luminal phosphate in hepatic microsomes from a patient with type 1c GSD (Nordlie *et al* 1992). The conclusion drawn in this paper is that there is more than one microsomal protein for transporting phosphate from the lumen of the ER, and that pyrophosphate counter-transport does not necessarily occur through all these proteins. This data could be interpreted as suggesting that many if not all of the transport proteins of the glucose-6-phosphatase system are involved in other enzyme processes also. The demonstration of a glucose-6-phosphate dehydrogenase isoenzyme in the lumen of microsomes (Hino & Minakami 1982) which presumably requires a transmembrane

glucose-6-phosphate transporter is also in favour of this. If T1 is necessary for G6PDH activity then the neutrophil defects seen in type 1b GSD patients may be (at least in part) be due to inactivity of G6PDH.

Ian Waddell has also performed painstaking topographical studies of the glucose-6-phosphatase system in the microsomal membrane which pulls together all the known data regarding glucose-6-phosphatase and confirms the existence of a number proteins associated with glucose-6-phosphatase activity on both surfaces of the ER membrane (Waddell & Burchell 1991). However the data I have presented on the activating effect of calcium on glucose-6-phosphate transport raises the possibility that calcium binding to the stabilising protein induces a conformational change in it or the other closely associated proteins of the glucose-6-phosphatase system thereby increasing their functional capacity. This links together the multicomponent and conformational theories of glucose-6-phosphatase function as presented in Chapter 1. It should be noted that this reconciliatory theory was first proposed in 1981 (Nordlie *et al* 1981) in an attempt to bring the then mutually exclusive ideas together and pool the investigational resources of the two factions.

In summary I have fallen a little short of two of the objectives I set out with: purifying the glucose-6-phosphate transport protein and developing an assay of glucose-6-phosphatase in a blood fragment. In retrospect both of these aims were very ambitious for a 2 year project. However both of these lines of investigation have been explored as described above and I have paved the way for these problems to be solved.

I did, however, achieve many of my aims: I described in detail several cases of glycogenolytic enzyme defects diagnosed in adult patients. All these patients are unusual and two of them demonstrated unique hepatic glucose-6-phosphatase abnormalities. The cases give insight into the physiological role of glucose-6-phosphatase in the liver and pancreas where its role appears to be related to insulin

secretion. I also proved for the first time that intestinal mucosal cells from all parts of the gut accessible to endoscopy contain specific glucose-6-phosphatase activity. I demonstrated that the activity in this tissue is too low and is too susceptible to proteolysis to allow diagnoses of glucose-6-phosphatase abnormalities to be made. The biochemical mechanism of microsomal glucose-6-phosphate transport was shown to involve sodium co-transport and require calcium, although the number of proteins involved remains to be clarified. For the first time also I was able to demonstrate that it is possible to pharmacologically alter T1 capacity thus raising the possibility of a novel drug treatment for diabetes mellitus and a drug treatment for the described cases with hypoglycaemia which can increase hepatic glucose output.

APPENDIX A

COPIES OF PUBLISHED PAPERS

ARISING FROM THIS THESIS

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Glycogen Storage Disease Diagnosed in Adults

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SUMMARY

Glycogen storage diseases are usually identified in childhood. We present the clinical, biochemical and histological features of 10 patients first diagnosed in adult life. Five had glycogen storage disease type 1a, one type 1c, two type IX, and in two patients there were previously unreported abnormalities of hepatic glucose-6-phosphatase system activity. Of the latter, one patient had an inhibitor of liver glucose-6-phosphatase (pseudo-1b glycogen storage disease) the other having abnormal glucose-6-phosphatase activity and microsomal pyrophosphate transport. A glucagon test is suggested as a useful screening procedure. Glycogen storage disease should be considered in adults with symptoms suggesting hypoglycaemia.

INTRODUCTION

The first instance of a specific enzyme abnormality causing a glycogen storage disease was reported in 1952 by Cori and Cori [1] and was shown to be due to glucose-6-phosphatase deficiency. Since that time, a number of other congenital disorders of enzymes involved in glycogen metabolism have been recognized. The glycogen storage diseases are usually thought to be identified only early in childhood: we report 10 adults in whom we have demonstrated abnormalities of hepatic glucose production. Their diagnosis over a short period of time in one Health Board region (approximate population 400,000) suggests that disorders of hepatic glycogen metabolism are probably less rare than had previously been recognized.

CASE REPORTS

Patient 1 presented at the age of 54 years with a severe metabolic acidosis (plasma lactate 12.5 mmol), profound hypoglycaemia (1 mmol/l), massive hepatomegaly and deranged liver function tests (Table 1) and blood clotting (prolonged bleeding time but normal KCCT and prothrombin time). Hepatomegaly had been noted at age 7 years and a bleeding diathesis shortly after. He developed gout at age 38. Two siblings died in infancy; three others were well. He had always been mentally 'slow'. Liver ultrasound suggested a large hepatocellular

TABLE 1. *Clinical details of patients*

Patient	Age/sex (years)	Symptom of hypoglycaemia	Hepatomegaly	LFT			KCCT (Sec/control)	PT	Fasting lactate (mmol/l)	48 hour fasting glucose (mmol/l)	Glycogen storage disease type
				BIL (umol/l)	AST (ul/l)	AP (u/l)					
1	54/M	+	+	27	747	2124	43/43	14/14	12.5	ND	1a
2	22/F	+	−	15	14	99	51/44	14.5/13	0.5	ND	1a partial
3	52/F	+	−	6	18	98	62/45	13.5/14	1	3.1	1a partial
4	54/F	+	+	5	87	67	43/46	16/15	ND	ND	1a
5	51/F	+	−	10	18	76	ND	ND	0.8	3.3	1a partial
6	51/M	−	+	13	32	175	51/43	15/13.5	1.4	ND	1c
7	20/F	+	−	7	27	102	42/49	15/15	ND	3.7	1 unclassified
8	47/M	+	−	18	18	106	54/47	15.5/14.5	ND	3.7	pseudo 1b
9	37/F	+	−	9	19	79	45/48	15.5/14	7.3	4.1	IX (partial)
10	18/F	+	−	13	13	73	53/49	15.5/15	ND	3.4	IX (partial)

Abbreviations: LFT, liver function tests; BIL, bilirubin; AST, aspartate transaminase; AP, alkaline phosphatase; ND, no data.

carcinoma (confirmed by liver biopsy). Serum α-fetoprotein was undetectable. Biopsy of noncancerous liver indicated an underlying type 1a glycogen storage disease.

Patient 2 had a recent history of hypoglycaemic symptoms exacerbated by exercise and relieved by food. She had been born prematurely and had required frequent day and night feeds in early infancy. She was given food frequently until she left home when she ate less often and symptoms began. Glucagon test showed a reduced glucose response (Table 2). Prothrombin time was normal but KCCT was prolonged (Table 1). A liver biopsy revealed a partial type 1a glycogen storage disease. The patient's symptoms resolved with regular meals containing cornstarch.

Patient 3 complained of episodes of sudden tiredness in the mid-afternoon exacerbated by activity and by the absence of a midday meal. The symptoms, which were relieved by chocolate, had been occurring for many years and had worsened after the menopause. There was a blunted blood glucose response to glucagon (Table 2) but no hypoglycaemia during a 48-hour fast conducted in hospital. Prothrombin time was normal but KCCT was prolonged (Table 1) due to a partial deficiency of Factor XI. Liver biopsy revealed a partial type 1a glycogen storage disease. She had no family history of similar symptoms and an uneventful obstetric history. Her symptoms were helped by cornstarch, regular meals and post-menopausal hormone replacement therapy.

Patient 4 noted post-menopausal sweating for 2 years associated occasionally with syncope. These episodes were precipitated by exercise and could be prevented by ingestion of carbohydrates. Some 11 years previously she had had radiotherapy for cervical carcinoma but recent cervical smears were class 2, showing mild dyskaryosis only. Liver function tests were abnormal (Table 1) but clotting tests were normal and she was hepatitis B surface antigen negative. An isotope liver scan showed an enlarged liver with uniform uptake. The glucagon test revealed a blunted glucose response (Table 2) and liver biopsy indicated a type 1a glycogen storage disease. Treatment with cornstarch, regular meals and post-menopausal hormone replacement therapy relieved her symptoms.

Patient 5 had a 13-year history of episodic weakness, especially of her legs, associated with incoordination, light headedness and tremulousness. These episodes were invariably associated with exercise and relieved by glucose tablets. Their frequency had increased since the menopause. Clotting tests were normal as were liver function tests (Table 1). A 48-hour

TABLE 2. *Results of glucagon tests*

Patient	Type of glycogen storage disease	Basal plasma glucose (mmol/l)	Peak	Rise in blood glucose	Time to peak (mins)
1	1a	ND			
2	Partial 1a	4.4	7.4	3	60
3	Partial 1a	5.2	7.4	2.2	30
4	1a	5.6	9.1	3.5	60
5	Partial 1a	5.3	7.4	2.1	30
6	1c	5.9	11.1	5.2	60
7	Partial 1 u/c	4.6	6.1	1.5	30
8	Pseudo 1b	4.6	6.6	2	30
9	Partial IX	4.7	6.9	2.2	30
0	Partial IX	4.5	6.1	1.6	30

1 mg Glucagon was given intramuscularly. Results are venous plasma glucose levels in mmol/l. Normal rise > 4 mmol/l in blood glucose level, usually at 30 minutes.

ND: glucagon test not performed; U/C: type 1 disease, abnormality unclassified.

TABLE 3. *Results of oral glucose tolerance tests*

Patient	Fasting	Time (mins)			
		30	60	90	120
* 1	3	6	6.9	5	3.7
2	4.5	7.4	9.4	9.4	8.5
5	4.1	5	3.9	3.6	3.5
6	5.6	11.6	13	9.8	6.8
7	4.2	10.1	12.2	12.2	9.3
8	4	2	<0.5	2.6	2.7
9	4.4	7.1	6.9	5.2	NS
10	4.6	7.9	6.7	7.4	9.1

75 g Glucose given orally after 10 hour overnight fast. Results are venous plasma glucose levels in mmol/l.

* GTT performed in 1966. Amount of glucose given not known.

NS = No sample.

Patient 6 Insulin 30, 54, 105, 5 mU/l at 0, 30, 60, 90, 120 mins. Patient 8 Insulin 11, 720, 108, 53, 36 mU/l at 0, 30, 60, 90, 120 mins.

fast did not precipitate the symptoms (Table 1) and a glucagon test indicated a blunted glucose response (Table 2). Liver biopsy revealed a partial type 1a glycogen storage disease. Frequent meals with additional cornstarch reduced the frequency of hypoglycaemia, and subsequent post-menopausal hormone replacement therapy further relieved the symptoms. The patient's mother and sister had similar symptoms.

Patient 6 had a past history of heavy alcohol consumption (30 units/day) but had abstained for 2 years, despite which his liver enzyme levels had remained elevated and he had a palpable liver. He complained of polyuria and glycosuria. His glucose tolerance test (Table 3) had a diabetic pattern. Clotting tests were normal as was the blood glucose response to glucagon (Table 2). Liver biopsy showed fatty change and a high glycogen content. Subsequent biochemical analysis revealed type 1c glycogen storage disease. Blood glucose control was satisfactory on a diabetic weight-reducing diet and he never developed symptoms of hypoglycaemia. Three years after diagnosis he developed a painful lytic lesion in the left acromion process. Biopsy revealed a metastatic deposit from a bronchogenic primary tumour and bone scan indicated widespread bony metastases from which he subsequently died. He had one brother who died at 3 weeks of age, but three other siblings remain healthy.

Patient 7 developed hypoglycaemic symptoms when she left home to enter university and began to eat less regularly. The symptoms usually occurred before meals and were relieved by eating. During some of these attacks, blood sugar levels of < 2 mmol/l had been recorded. A 48-hour fast was not associated with hypoglycaemia (Table 1). Clotting studies were normal, as were liver function tests. Glucose response to glucagon was blunted and she exhibited an impaired glucose tolerance test. Liver biopsy showed lymphocytic infiltration suggestive of a viral illness but there was no serological evidence to support this. Enzymatic analysis showed abnormally low activity of the glucose-6-phosphatase enzyme and very low transport capacity of the transport protein T2, similar to that previously found by us in human liver samples early in gestation [2] and is without defined classification. It is of interest

that this patient had been born 3 weeks prematurely. Symptoms resolved with regular polycarbohydrate meals.

Patient 8 had a 5-year history at presentation of hypoglycaemia which occurred shortly after the ingestion of glucose or sucrose. A 48-hour fast did not precipitate hypoglycaemia (Table 1). A fasting glucagon test showed a blunted glucose response (Table 2) and an intravenous glucose tolerance test resulted in hypoglycaemia with a blood glucose of 1.1 mmol/l at 90 min following a large insulin rise (peak level of 1520 mU/l at 60 min and absent glucagon rise (peak <11 pg/ml). An oral glucose tolerance test also induced hypoglycaemia some 60 min post ingestion (Table 3) associated with a similar rise in insulin to >750 mU/l. Liver biopsy indicated some lymphocytic histiocytic infiltration but no evidence of lymphoma. Enzymatic analysis of the liver tissue biopsy revealed the presence of an inhibitor of T1, the glucose-6-phosphate transport protein of the glucose-6-phosphatase system, in the patient's liver. This is similar to the pattern of glucose-6-phosphatase seen in some infants and is named pseudo-type 1b glycogen storage disease. Glycogen stores were normal. Treatment was aimed at reducing the insulin peak response to glucose by diazoxide therapy. Octreotide was also effective in reducing the hyperinsulinaemia.

Patient 9 had a 3-year history of episodes of hunger followed by loss of memory, uncontrollable upper limb jerking and occasional syncope. Her symptoms worsened when she began working and began eating less frequently. Liver function tests, clotting studies and EEG were normal. A 48-hour fast did not precipitate hypoglycaemia (Table 1), but her glucose response to glucagon was blunted. Liver biopsy indicated excess glycogen, but the glucose-6-phosphatase assay showed all parts of the system to be present in normal proportions, although to a slightly reduced degree. A muscle biopsy was undertaken to further identify the abnormality: histology was normal. Biochemical analysis of liver and muscle indicated reduced phosphorylase kinase activity indicative of partial type IX glycogen storage disease. The patient's symptoms responded to regular polycarbohydrate meals. Her obstetric history was unremarkable.

Patient 10, the daughter of *Patient 9*, suffered episodic dizziness and light headedness before a midday meal, especially if she had not had breakfast, relieved by eating. The results of an oral glucose tolerance test were unusual, with a double peak of glucose levels (Table 3). The glucose response to glucagon was blunted (Table 2). Liver and skeletal muscle histology were unremarkable, but biochemical analysis indicated reduced phosphorylase kinase activity of partial type IX glycogen storage disease. The patient's symptoms responded to dietary manipulation.

Brief data on *Patients 1*, *2*, *5* and *6* are given elsewhere [3]. *Patient 8* is discussed in reference [4].

METHODS

Endocrine Testing

A 75 g oral glucose tolerance test was performed with patients in the fasting state. Glucagon tests used 1 mg glucagon (Novo Laboratories) given intramuscularly, blood samples being taken for glucose estimation 0, 30, 60 and 90 minutes later. Normal blood glucose rise was taken as >4 mmol/l above the fasting level. The analysis was undertaken using a Beckman glucose analyser. Total serum insulin was measured by radioimmunoassay (MAIA, Serono Diagnostics, Woking, UK).

Biopsies

Liver biopsies were obtained under local anaesthesia unless otherwise indicated using Surecut needles (TSK Laboratories, Japan). Samples for biochemical analysis were maintained in ice-cold 0.25 M sucrose/5 mM HEPES buffer, pH 7.4 (SH buffer). Skeletal muscle biopsies (*Patients 5*, *9* and *10*) were taken from the tibialis anterior under local anaesthesia using Tilley-Henkel forceps. Biopsies were transported fresh for histology and in SH buffer for biochemical analysis.

Biochemical Analysis of Biopsies

Assays were performed on fresh (unfrozen) tissue [5]. Tissue samples were homogenized by hand in SH buffer using Jencons glass homogenizers to a final volume 10 times the original tissue weight. Homogenates were centrifuged ($10,000 \times g$ for 1 min) and phosphorylase kinase activity in the supernatant was determined where indicated. The supernatant was spun again ($105,000 \times g$ for 1 hour at 4°C) and the resulting microsomal pellet was resuspended in SH buffer using a Jencons glass homogenizer to a final volume equal to the wet tissue weight. The final suspension contained the microsomal fraction used for glucose-6-phosphatase assay.

Glucose-6-phosphatase, mannose-6-phosphatase and pyrophosphatase activities were assayed as described previously [6], but modified by the use of 0.1 M HEPES pH 6.5 as buffer in the substrates, glucose-6-phosphate concentrations of 1, 1.4, 2, 2.6, 5 and 30 mM and pyrophosphate concentrations of 0.5, 1, 1.4, 2, 2.6 and 5 mM. Microsomal intactness was estimated and corrected for using mannose-6-phosphatase activity and UDP glucuronosyl transferase activity towards 1-naphthol [5, 6]. Total phosphorylase (a + b) activity was assayed in the direction of glycogen synthesis in the presence of 5′-AMP [7]. Phosphorylase

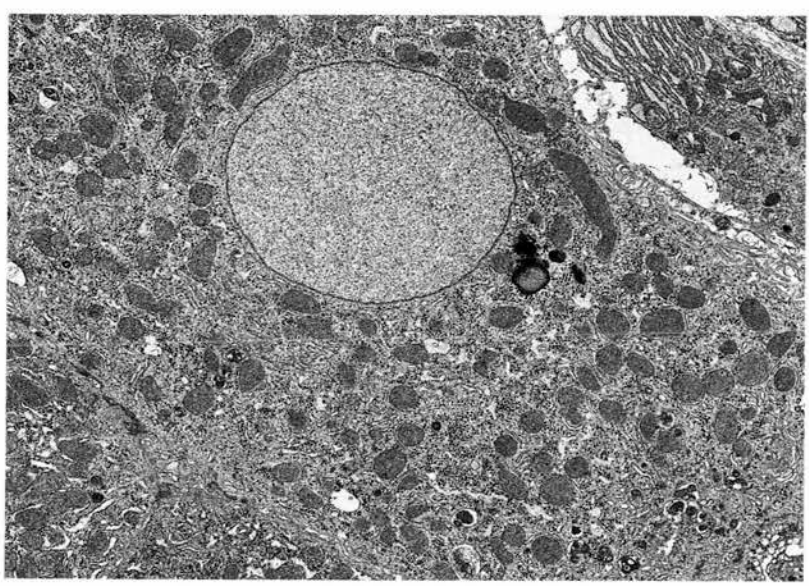

FIG. 1. Electron micrograph of control liver. Magnification ×4000. Normal glycogen stores (black speckles) between organelles which are distributed throughout the hepatocytes.

kinase activity was measured by the methods of Krebs as modified by Cohen [8]. Glycogen levels were assayed as previously described [9] and protein levels by the method of Peterson [10].

Light microscopy was undertaken on tissue fixed on phosphate-buffered formaldehyde/phenol-formaldehyde [11]. Paraffin sections were stained with haematoxylin and eosin and PAS [12]. For electron microscopy the tissue was fixed in 0.2 M cacodylate-buffered 3 per cent glutaraldehyde and embedded in Epon. Thin sections were stained with uranylacetate and lead citrate and examined with a JEOL 100 CX electron microscope at 60 kV. An electronmicrograph of a control liver section is shown is Fig. 1.

Immunoblot and immunodot analysis. SDS/PAGE was carried out in a 7–16 per cent (w/v) polyacrylamide gels as described by Laemmli [13]. Proteins separated on SDS/polyacrylamide gels were electrophoretically transferred to nitrocellulose, as described by Towbin *et al.* [14]. The Western blot and dot blots were immunostained with antibodies previously shown to be monospecific for the catalytic subunit of glucose-6-phosphatase or for T2 the microsomal phosphate/pyrophosphate transport protein [2, 15]. Immunoreactive polypeptides were detected using a biotin-streptavidin-peroxidase linked detection system with 4-chloro-1-naphthol as the substrate [16]. In the immunodot experiments human liver cytosol was used as a negative control since it does not contain the components of the microsomal glucose-6-phosphatase system.

RESULTS

Liver Histochemical and Electron Microscopic Features

In normal human adult liver biopsy samples ($n=60$) glycogen levels are always <40 mg/g liver (Table 4): *Patients 1–7, 9* and *10*, but not *Patient 8* had abnormally high glycogen levels,

TABLE 4. *Glucose-6-phosphatase activity in hepatic biopsy specimens*

Patient	Intact microsomes		Disrupted microsomes		Glycogen content (mg glycogen/g wet weight liver)
	Vmax (μmol/min/mg)	Km (mM)	Vmax (μmol/min/mg)	Km (mM)	
1	0	ND	0	ND	134
2	0.01	1.7	0.02	1.1	70
3	0.01	3.3	0.04	1	82
4	0	ND	0	ND	166
5	0.03	3.3	0.08	1.3	96
6	0.09	6.9	0.53	1.1	80
7	0.01	2.2	0.06	1	54
8	0.03	16.2	0.2	0.9	28
	0.15*	2.2	0.2	0.9	
9	0.16	2.1	0.3	0.4	70
10	0.1	2.7	0.14	0.4	62
Mean control values (n=60)	0.23 ± 0.02	2.6 ± 0.2	0.35 ± 0.03	0.8 ± 0.08	<40

ND = Not determinable.
* Microsomes incubated with 1% bovine serum albumin.

TABLE 5. *Histological examination of biopsies from patients 1–10*

Patient number	Comment	Glycogen content seen by PAS*
1	Hepatocellular carcinoma. Massive glycogen store in remaining liver	3+
2	Normal liver	+
3	Normal liver	+
4	Marked fatty change in liver. Portal fibrosis (moderate)	+
	Moderate chronic active inflammatory infiltrate	+
5	Increased perivenular lipofuscin in liver	3+
6	Mild fatty change in liver. Increase in portal tract lymphocytes	+
7	Excess lymphocytes in portal tracts of liver	3+
8	Mixed lymphocytic histiocytic infiltration	3+
9	Liver and muscle both normal	+
10	Liver and muscle both normal	3+

* + Represents normal glycogen stores; 3+ markedly increased glycogen stores.

in the range previously found in liver from patients with glycogen storage disease [3, 17–19]. Glycogen content of liver samples as assessed by routine histological PAS staining (Table 5) did not always correlate well with the biochemical determination of liver glycogen, presumably due to the loss of glycogen into the formaldehyde during tissue processing. However, the electron microscopic appearance of the liver samples correlated well with the biochemical determinations. Ultrastructural abnormalities seen by electron microscopy in the livers of these patients ranged from mild excess of glycogen in the cytoplasm to increased

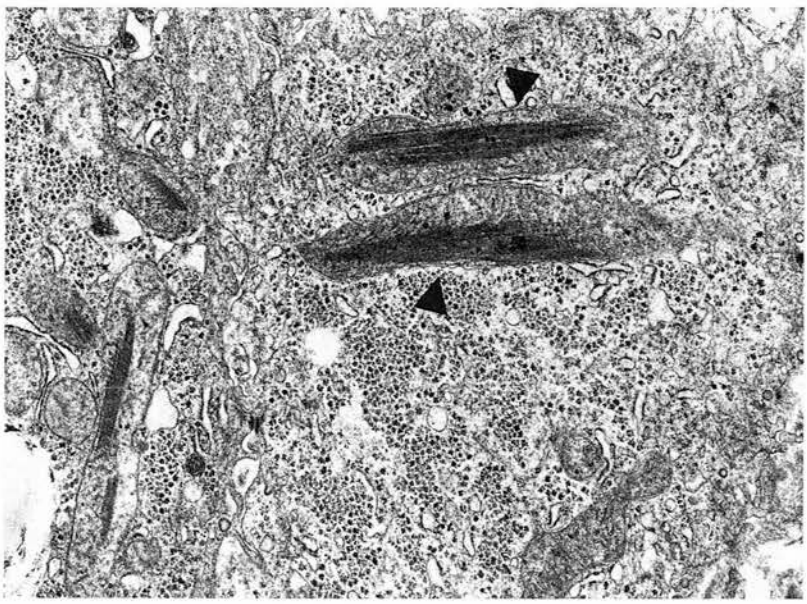

FIG. 2. Electron micrograph of liver biopsy from *Patient 4*. Magnification × 10,000. Glycogen stores are abundant and the two mitochondria in the centre of the field contain paracrystalline arrays (which are indicated by arrows).

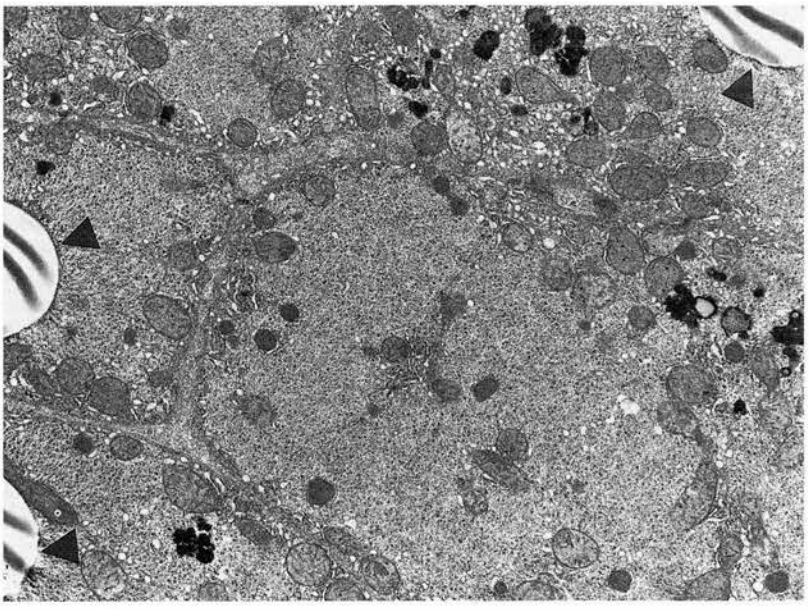

FIG. 3. Electron micrograph of liver biopsy from *Patient 5*. Magnification $\times 5000$. Hepatocytes contain marked excess of glycogen which has displaced the organelles to the peripheries of the cells. Lipid droples are also seen.

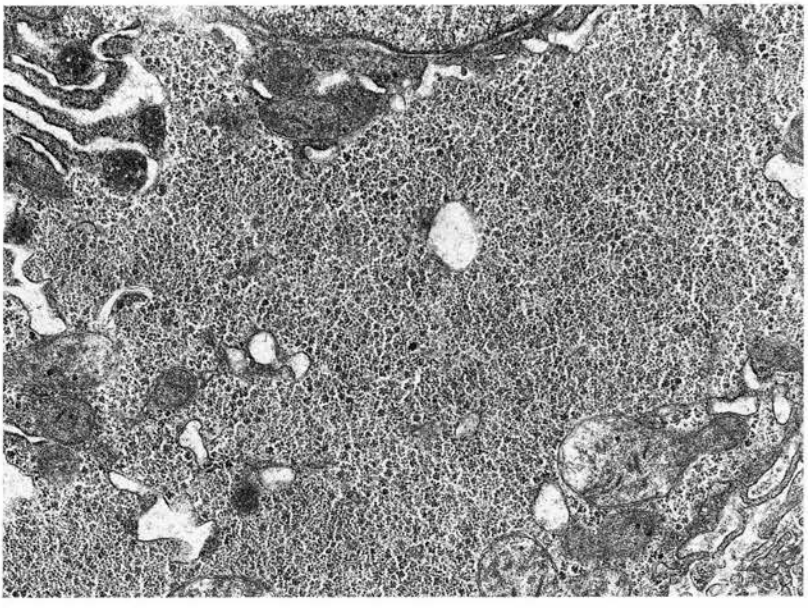

FIG. 4. Electron micrograph of liver biopsy from *Patient 5*. Magnification $\times 10{,}000$. This plate is a field from Fig. 4 and shows more clearly the large amount of stored glycogen (indicated by arrows).

amounts displacing the organelles in the hepatocytes (Figs. 1–4). Other non-specific findings were of lipid droplets and mitochondrial paracrystalline arrays. The liver biopsy of *Patient 8* showed mixed lymphocytic histiocytic infiltration initially suggesting lymphoma. CT scanning showed no evidence of lymphoma, haematological findings were normal and the patient is now well. A subsequent review of liver biopsies of patients with lymphoma in Dundee has not shown an increase in glycogen stores. Apart from *Patient 1* (hepatocellular carcinoma) and *Patient 6* (fatty liver of diabetes) the liver biopsy histology was non-specific (Table 5).

Liver Glucose-6-Phosphatase Kinetics and Immunochemistry

The kinetics of glucose-6-phosphatase activities using glucose-6-phosphate as substrate are shown in Table 4. Disruption of microsomes removes the controlling influence of the transport proteins of the glucose-6-phosphatase system and is a direct measure of the glucose-6-phosphatase enzyme itself. In *Patients 1* and *4* the glucose-6-phosphatase enzyme activity was absent, indicating type 1a glycogen storage disease. The defect in these patients cannot, however, be the same as immunological analysis revealed the complete absence of the glucose-6-phosphatase enzyme in *Patient 1* (Fig. 6, d6), whereas normal amounts of apparently normal molecular weight enzyme protein were present in *Patient 4*. *Patients 2*, *3* and *5* all had some glucose-6-phosphatase enzyme activity, but with an abnormally low V_{max} and normal K_m in both intact and disrupted microsomes, indicating partial type 1a glycogen storage disease (Table 4). In all three patients immunological analysis revealed normal amounts of normal molecular weight glucose-6-phosphatase enzyme protein (Fig. 6, d4, d7, e centre). In *Patients 1–5* all of the other components of the glucose-6-phosphatase system were normal (Fig. 6, f, 2, 6 and 7). *Patient 7* also had abnormally low glucose-6-phosphatase enzyme activity (Table 4) with a normal K_m in intact and disrupted microsomes. The situation was quite different from that of *Patients 2*, *3* and *5* since abnormally low levels of the glucose-6-phosphatase enzyme were found on immunoblot analysis (Fig. 6a, lane 1 and e4; note much more microsomal protein was loaded than for other samples in order to distinguish clearly whether or not even low levels of protein were present). In addition, the activity of the glucose-6-phosphatase enzyme in intact microsomes with pyrophosphate as substrate (Table 6) was also abnormally low in this patient, indicating low transport capacity of T2, the phosphate/pyrophosphate transport protein of the glucose-6-phosphatase system (see Fig. 5). The pattern of activity and protein levels in this patient are very similar to those

TABLE 6. *Pyrophosphatase activity in hepatic biopsy specimens*

Patient	Intact microsomes		Disrupted microsomes	
	Vmax (μmol/min/mg)	Km (mM)	Vmax (μmol/min/mg)	Km (mM)
1	0	ND	0	ND
5	0.01	3.2	0.04	2.3
6	0	ND	0.29	1.9
7	0.004	1.7	0.07	1
Mean control values (n = 25)	0.08 ± 0.001	2.5 ± 0.32	0.24 ± 0.02	0.7 ± 0.1

ND = Not determinable.

seen previously early in human gestation [2] so a regulatory developmental defect cannot be ruled out.

Patient 6 had normal glucose-6-phosphatase enzyme activity in disrupted microsomes but an abnormally high Km in intact microsomes (Table 4), indicating a defect in one of the transport proteins. To determine which transport protein was involved, pyrophosphatase activity was measured (Table 6). The lack of any activity in intact microsomes demonstrated that T_2, the phosphate/pyrophosphate transport protein, was deficient (type 1c glycogen storage disease). Immunoblot analysis revealed the complete absence of the T2 protein in this patient (Figure 6, b2 and f5). *Patient 8* had normal glucose-6-phosphatase enzyme activity in disrupted microsomes (Table 4) but a very high K_m in intact microsomes, indicating a transport protein defect. However, this patient does not have a genetic deficiency of the glucose-6-phosphatase system: an inhibitor of the T1 protein (the glucose-6-phosphate transport protein, see Fig. 5) was found in the liver of this patient, and the enzyme activity could be restored to normal by incubating microsomes in the presence of bovine serum albumin (Table 4). The livers of *Patients 1–7*, *9* and *10* did not contain T1 inhibitor.

Liver and Muscle Phosphorylase Kinase Kinetics

Patients 9 and *10* had normal liver glucose-6-phosphatase enzyme activity (Table 4) and normal protein levels (Fig. 6). There is no glucose-6-phosphatase activity in normal skeletal muscle. In *Patient 9*, total phosphorylase a and b levels were within the normal range in both liver and muscle (0.5 U in liver, 1.6 U in muscle). However, phosphorylase kinase activity was reduced to approximately 10 per cent of normal in liver and muscle (at pH 6.8, = 0.008 U and muscle = 0.01 U). These data suggest a partial defect of liver and muscle phosphorylase kinase (partial type IX glycogen storage disease). Electromyography showed a slightly abnormal interference pattern, thought to be appropriate for a myopathic process (increase

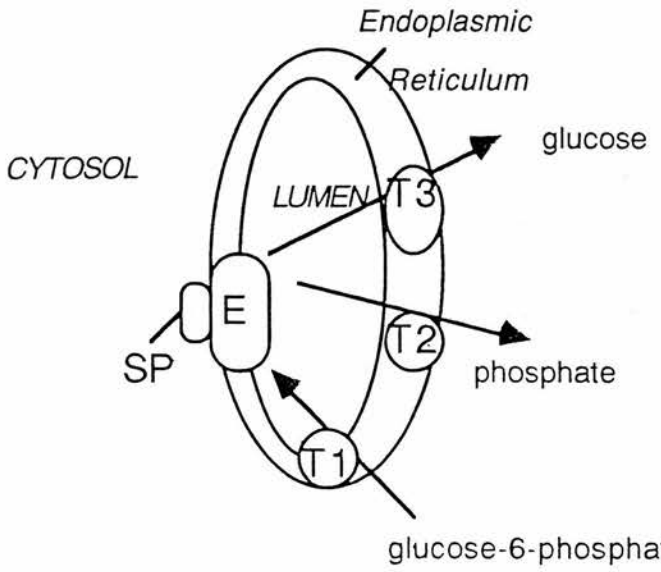

FIG. 5. Diagrammatic representation of the hepatic microsomal glucose-6-phosphatase system. Catalytic subunit (E); stabilising protein (SP); transport proteins for glucose-6-phosphate (T_1), phosphate (T_2) and glucose (T_3).

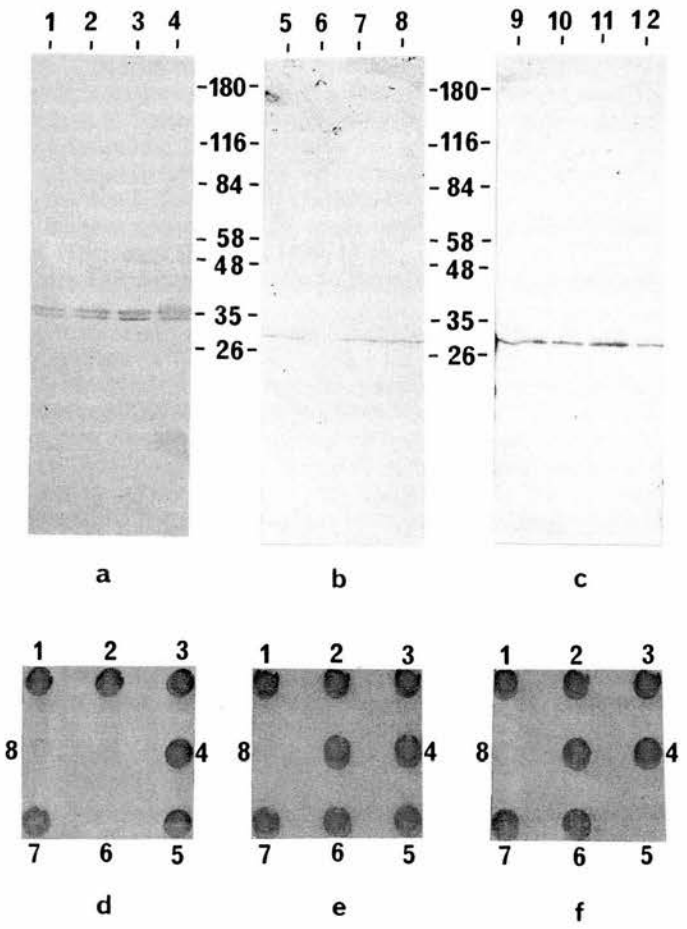

FIG. 6. Immunological analysis of human liver microsomes showing the presence or absence of the glucose-6-phosphatase catalytic subunit and T2 the microsomal phosphate/pyrophosphate transport protein.

(a) Immunoblot analysis using an anti glucose-6-phosphatase catalytic subunit antibody.
Lane 1—*Patient 7*—hepatic microsomes (40 μg);
Lane 2—*Patient 8*—hepatic microsomes (12 μg);
Lane 3—*Patient 9*—hepatic microsomes (12 μg);
Lane 4—*Patient 10*—hepatic microsomes (12 μg);

(b) and (c) Immunoblot analysis using an anti T2 antibody.
Lane 5—*Patient 1*—hepatic microsomes (20 μg);
Lane 6—*Patient 6*—hepatic microsomes (80 μg);
Lane 7—*Patient 5*—hepatic microsomes (20 μg);
Lane 8—Control adult human hepatic microsomes (20 μg);
Lane 9—*Patient 7*—hepatic microsomes (21 μg);
Lane 10—*Patient 8*—hepatic microsomes (20 μg);
Lane 11—*Patient 9*—hepatic microsomes (19 μg);
Lane 12—*Patient 10*—hepatic microsomes (21 μg).

(d) and (e) Immunodot blot analysis using anti glucose-6-phosphatase antibody.
d1 Control rat hepatic microsomes (5 μg);
2 & 3 Control adult human hepatic microsomes (5 μg);
4 *Patient 2* hepatic microsomes (5 μg);
5 *Patient 6* hepatic microsomes (5 μg);
6 *Patient 1* hepatic microsomes (5 μg);
7 *Patient 5* hepatic microsomes (5 μg);
8 Control human hepatic cytosol (5 μg);
Centre Control microsomes from an infant with classical type 1a glycogen storage disease.

e1 Control rat hepatic microsomes (5 μg);
2 & 3 Control human adult hepatic microsomes (5 μg);
4 *Patient 7* hepatic microsomes (15 μg);
5 *Patient 8* hepatic microsomes (5 μg);
6 *Patient 9* hepatic microsomes (5 μg);
7 *Patient 10* hepatic microsomes (5 μg);
8 Control liver hepatic cytosol (5 μg);
Centre *Patient 3* hepatic microsomes (5 μg).

(f) Immunodot blot analysis using anti T2 antibody.
f1 Control rat liver microsomes (5 μg);
2 & 3 Control human adult hepatic microsomes (5 μg);
4 *Patient 2* hepatic microsomes (5 μg);
5 *Patient 6* hepatic microsomes (5 μg);
6 *Patient 1* hepatic microsomes (5 μg);
7 *Patient 5* hepatic microsomes (5 μg);
8 Control human hepatic cytosol (5 μg);
Centre Control microsomes from an infant with classical type 1a glycogen storage disease (5 μg).

in high frequent components). Biochemical analysis of biopsies from *Patient 10* showed high (liver) or high normal (skeletal muscle 19.9 mg/g) glycogen stores and low normal hepatic glucose-6-phosphatase activity. Total phosphorylase (a + b) activity was normal in liver and in muscle (1.1 and 5.3 units respectively). Phosphorylase kinase activity at pH 6.8 (0.09 u liver, 0.12 u muscle) and at pH 8.6 (0.11 u liver, 0.23 u muscle) were within 'normal ranges', but at these levels the assay cannot unfortunately distinguish 'half normal', from 'normal'. In view of the history, the evidence of glycogen accumulation and her mother's abnormalities, a diagnosis of partial type IXc glycogen storage disease was made.

Patients 9 and *10* are also very unusual in that type IXc glycogen storage disease, in which both hepatic and skeletal muscle phosphorylase kinase are deficient, has been thought to be inherited in an autosomal recessive manner [20, 21]—very unlikely in mother and daughter. A sibling of *Patient 10* also had a blunted glucagon test but is asymptomatic and liver biopsy has, therefore, not been undertaken.

DISCUSSION

We have diagnosed a variety of defects leading to type 1 glycogen storage disease in adults, as well as two cases of type IX glycogen storage disease. Although there have been a few reports of type 1 glycogen storage disease in adults [22, 23] this condition is generally thought of as a disease that is diagnosed early in childhood [24, 25]. Some of the patients, for example, *Patient 1*, might reasonably have been expected to be diagnosed in early childhood but many had such mild symptoms in earlier years that further investigation was not undertaken.

Until recently a large wedge liver biopsy would have been needed to diagnose such deficiencies; the microtechniques used in these studies now make it possible to diagnose type 1 glycogen storage disease deficiencies using a needle liver biopsy taken under local anaesthesia. This makes it probable that many more of the milder deficiencies will be

diagnosed in adults in the future. The recent purification and cloning of several of the protein components of glucose-6-phosphatase (see [26–28] for recent reviews) will also lead to easier diagnosis of type 1a, 1aSP, 1b, 1c and 1d glycogen storage diseases in all patients.

All of the patients (except *Patient 6*) had symptoms which could be attributed to hypoglycaemia. In only three of the patients was hypoglycaemia proven biochemically, even during exercise stress undertaken after a 48-hour fast. The lack of proven hypoglycaemic episodes in many type 1 glycogen storage disease patients, even in those with classic type 1 glycogen storage disease diagnosed early in childhood, is well recognized [29–34].

In patients with hypoglycaemia of uncertain aetiology the value of the glucagon test (1 mg administered intramuscularly) as a screening procedure is clear. Only one of the nine patients in whom the test was performed had an increase in blood glucose >4 mmol/l above fasting. This patient also had no symptoms of hypoglycaemia and was indeed diabetic (in keeping with the first case of type 1c glycogen storage disease described [35]). We have used an increase in blood glucose of 4 mmol/l above fasting as a cut-off, as suggested by Fernandes *et al.* [36] rather than 2 mmol/l as advocated by Dunger and Leonard [37] as we are screening for partial, less severe defects in glycogen metabolism than those described in infants with complete enzyme abnormalities. Conversely it appears that the oral glucose tolerance test is of little diagnostic benefit, being normal in seven of 10 of our patients.

The diagnosis of partial glycogen storage diseases is unlikely to be aided by routine histopathological examination of liver biopsies as provided in most centres. There may be large discrepancies in estimation of glycogen content of a sample between PAS staining, electron microscopy and the biochemical method (described in [9]) due to its loss into the formaldehyde and during tissue processing. However, electron microscopic examination of liver samples may be helpful and we did find a correlation between the ultrastructural estimation of glycogen stored and the biochemical estimates.

These patients indicate that partial defects in hepatic glycogen-metabolizing enzymes are probably less rare in the adult population than has previously been thought and we recommend that patients with symptoms which suggest hypoglycaemia should be screened with a glucagon test as described, even if hypoglycaemia cannot be proven. If the glucagon test is abnormal, such patients should undergo a needle liver biopsy at a centre capable of performing the necessary biochemical analyses. The diagnosis in patients such as these cannot be made on routine histological examination of liver samples but must rely on analysis in laboratories regularly carrying out the required complicated assays.

ACKNOWLEDGEMENTS

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Reactive Hypoglycaemia in Association with Disordered Islet Function and Abnormal Hepatic Glucose-6-Phosphatase Activity: Response to Diazoxide

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Severe reactive hypoglycaemia was confirmed in a non-diabetic male patient by a counter-regulatory hormone (GH, cortisol and catecholamine) response to profound hypoglycaemia induced by an intravenous glucose load. There was also evidence of disordered pancreatic islet cell paracrine regulation with hyperinsulinaemia and absent glucagon response to hypoglycaemia. A defect in the patient's hepatic glucose-6-phosphatase enzyme system was documented. Because of severe symptoms, dietary control was insufficient, but the patient responded clinically and biochemically to 18 months of oral diazoxide therapy. He also showed good biochemical response to a single dose (100 μg IM) of the somatostatin analogue octreotide.

KEY WORDS **Reactive hypoglycaemia Hyperinsulinaemia Glucose-6-phosphatase Diazoxide Somatostatin Octreotide**

Introduction

Reactive (postprandial) hypoglycaemia is an uncommon disorder comprising hypoglycaemic symptoms usually occurring 1 to 4 h after a meal. It can be caused by gastrointestinal disease, hormonal abnormalities, and rarely by deficiency of fructose 1:6 diphosphatase, a hepatic gluconeogenic enzyme.[1] In many patients, however, a precise cause is never established. The management of reactive hypoglycaemia is based on small meals low in refined carbohydrate, and several drugs have been tried where dietary management is insufficient.[2] We report a case of severe reactive hypoglycaemia involving both abnormalities of pancreatic islet hormone secretion and an unreported abnormality of hepatic glucose-6-phosphatase activity, with clinical response to two drugs not previously used in treating reactive hypoglycaemia.

Case Report

A Caucasian farmer led a very active working and sporting life, playing rugby and cricket into his thirties. He presented aged 42 years with neuroglycopenic and autonomic symptoms of hypoglycaemia, worst between 1000 and 1030 h after an early rise (0500 h) and heavy breakfast (0900 h). These symptoms were precipitated by physical activity and were relieved by the ingestion of food. There was no past medical history or family history of note. He did not smoke and alcohol consumption was < 1 U day^{-1}. Physical examination was unremarkable.

A prolonged fast showed a 48-h insulin:glucose ratio of 0.12 mU mmol^{-1} (normal <0.3 mU mmol^{-1}). A prolonged 75 g oral glucose tolerance test (OGTT) (Figure 1) produced symptomatic hypoglycaemia. A diagnosis of reactive hypoglycaemia was made and dietary advice given.

At age 47 years, he was re-referred with worsening hypoglycaemic symptoms in the same pattern as before. Other history and clinical examination were unremarkable. OGTT demonstrated a large rise in total serum insulin (peak 720 mU l^{-1}) (MAIA assay, Serono Diagnostics, Woking, UK) with a small rise in plasma glucose (Figure 2). An intravenous glucose tolerance test (IVGTT) (0.5 g kg-body-weight^{-1}) was performed. This produced severe, profound hypoglycaemia after 90 min, following a period of hyperinsulinaemia, and a virtually absent glucagon counter-response (Table 1). Other counter-regulatory hormone responses to hypoglycaemia were normal (including adrenaline and noradrenaline, peak levels of both 3 nmol l^{-1} at 120 min). The hypoglycaemia could only be reversed by small boluses of IV glucose until he was able to ingest carbohydrate. A short

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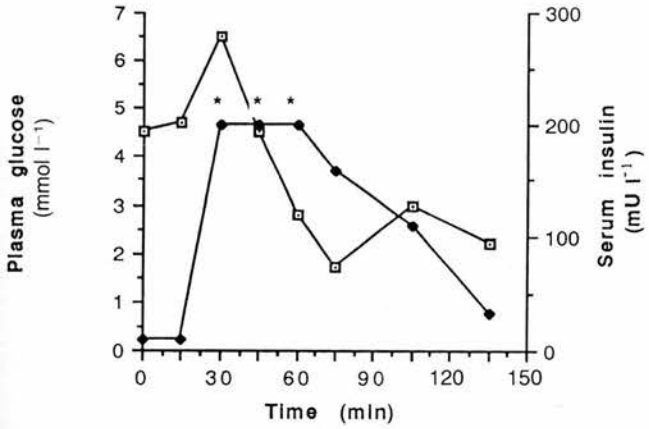


Figure 1. Extended OGTT in the patient aged 42 years with reactive hypoglycaemia; ⊡——⊡ plasma glucose, ◆——◆ serum insulin, * serum insulin levels given as >200 mU l^{-1}, further dilutions not performed

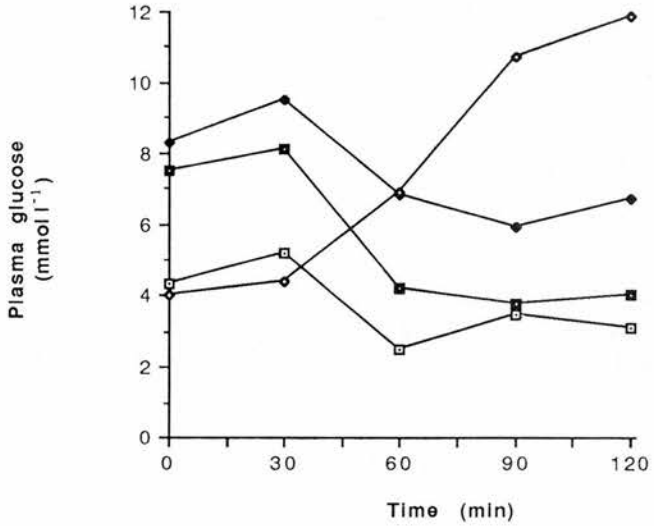
Table 1. Results of an intravenous GTT (0.5 g kg-body-weight^{-1}) in the patient with reactive hypoglycaemia

Time (min)	Plasma glucose (mmol l^{-1})	Serum insulin (mU l^{-1})	Serum growth hormone (mU l^{-1})	Serum cortisol (nmol l^{-1})	Serum glucagon (pmol l^{-1})
0	4.1	20	3.6	178	5
15	14.3	408	1.1	507	5
30	9.5	400	1.0	554	<5
45	6.6	479	0.9	537	5
60	3.5	1520	0.8	454	5
90	1.2	268	0.8	360	5
120	1.1	96	>62.5	885	11

ynacthen test (250 μg IM) was normal (baseline serum ortisol 355 nmol l^{-1}, peak 1000 nmol l^{-1}).

A fasting glucagon test (1 mg glucagon IM) showed a lunted rise in blood glucose levels (normal rise > 4 mmol l^{-1}) followed by hypoglycaemia (Figure 3). he test suggested abnormal hepatic glucose production o a needle liver biopsy was performed after an overnight ast. Liver histology was normal as was the measured lycogen content of 27.2 mg-glycogen g-liver-wet-veight^{-1} (normal < 40 mg g^{-1}). Hepatic glucose-6-hosphatase kinetics were measured on a microsomal ubfraction (Table 2).[3]

Assaying glucose-6-phosphatase activity in intact icrosomes gives a measure of the activity of the catalytic ubunit in conjunction with the three transport proteins igure 4). Kinetic analysis has shown that it is the lucose-6-phosphate (T_1) transporter which is rate-limiting o glucose-6-phosphatase activity in intact structures.[4] Disrupting the microsomes with histone allows direct neasure of catalytic subunit activity.[5] Microsomal prep-rations are heterogeneous and contain a portion of isrupted structures, the extent of disruption being



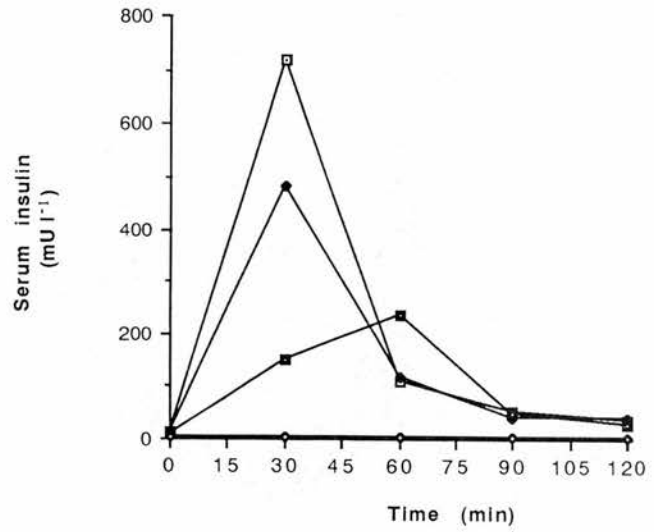


Figure 2. Plasma glucose and serum insulin concentrations during an OGTT in the patient with reactive hypoglycaemia (same patient as in Figure 1, now aged 47 years); ⊡——⊡ pre-treatment; ◆——◆ diazoxide 900 mg day^{-1}, ■——■ diazoxide 400 mg day^{-1}, ◇——◇ diazoxide + octreotide

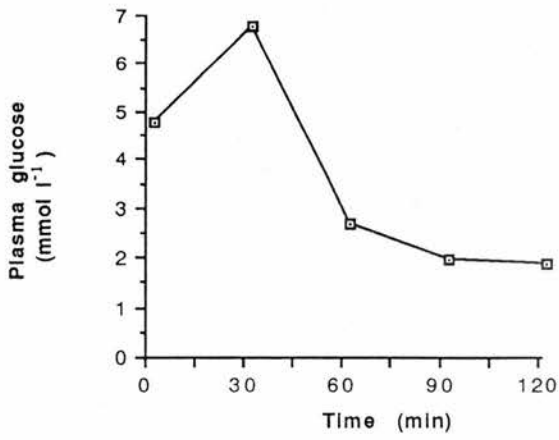


Figure 3. Results of fasting glucagon test (1 mg IM) in the patient with reactive hypoglycaemia

Table 2. Hepatic glucose-6-phosphatase activity in a hepatic microsomal preparation from the patient with reactive hypoglycaemia

	Intact microsomes		Disrupted microsomes	
	V_{max} (μmol min^{-1} mg^{-1})	K_m (mmol l^{-1})	V_{max} (μmol min^{-1} mg^{-1})	K_m (mmol l^{-1})
Patient	0.03	16.5	0.20	0.90
Patient + BSA[a]	0.15	2.2	0.20	0.90
Control[b]	0.26	2.3	0.49	0.80

[a]Bovine serum albumin solution 10 g l^{-1}.
[b]Control data from assaying glucose-6-phosphatase activity in adult liver biopsies (n = 40) taken for diagnostic reasons other than abnormalities of carbohydrate metabolism. Study approved by Tayside Health Board Ethical Committee.

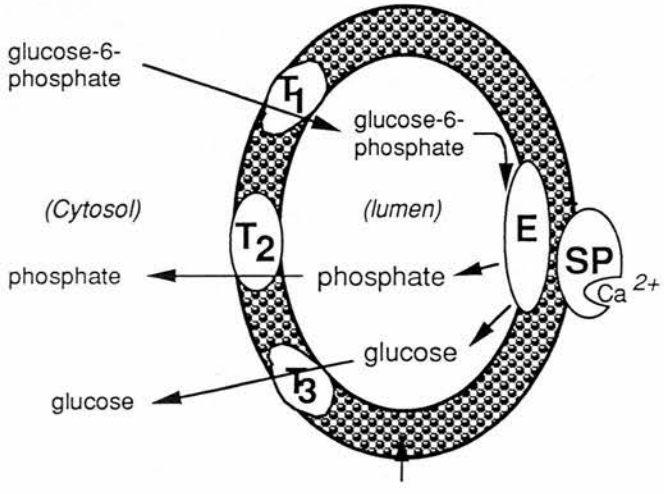


Figure 4. A diagrammatic representation of the hepatic microsomal glucose-6-phosphatase system. The catalytic subunit (E) lies on the luminal surface of the endoplasmic reticulum membrane (ERM) associated to a calcium-binding stabilizing protein (SP). There are three transport proteins, allowing the passage of substrate glucose-6-phosphate to the lumen of the ER (T_1), and the efflux of reaction products phosphate (T_2) and glucose (T_3)

calculated by assaying with mannose-6-phosphate which is a substrate of the catalytic subunit, but is not transported by T_1. The K_m of the intact microsome is inversely proportional to the affinity of T_1 for glucose-6-phosphate. Thus a high K_m indicates inhibition of T_1. Our patient's high K_m in intact microsomes, restored to normal by pre-incubating the microsomes with bovine serum albumin (BSA) 10 g l^{-1}, implies that there is a reversible inhibitor of T_1 binding to the transport protein in this patient's liver preparation. BSA is a non-specific binder of many ions and peptides and thus the nature of this inhibitor is not clear. The activity of the catalytic subunit (as indicated by the disrupted microsomal assays) is normal.

CT scan of the pancreas before and during rapi infusion of contrast medium did not reveal any abno mality.

In view of the marked hyperinsulinaemic response t glucose, treatment with oral diazoxide was commence at 900 mg day^{-1}. This gave good relief of hypoglycaemi symptoms within 14 days. Repeat OGTT performed afte 14 days of 900 mg day^{-1} diazoxide showed raised fastin and peak plasma glucose levels but a reduced rise i serum insulin concentration (peak 485 mU l^{-1}) (Figur 2).

The dose of diazoxide was reduced to 50 mg twic daily to alleviate side-effects (fluid retention) and the gradually increased to 200 mg twice a day with clinica improvement. OGTT was repeated 8 weeks after startin 400 mg day^{-1} diazoxide and, although fasting and pea plasma glucose levels were elevated compared wit levels prior to therapy, the peak rise in insulin wa reduced (Figure 2).

While on 400 mg day^{-1} diazoxide the patient wa given a single dose of 100 μg octreotide (Sandostatin Sandoz, Camberley, UK) IM and the OGTT repeated The insulin response to oral glucose was abolished (Figur 2), but the patient suffered abdominal discomfort. Thre years later the patient remains symptomatically well o diazoxide 200 mg twice daily and has octreotide at hom for emergency use (as yet unused).

Discussion

The diagnosis of reactive hypoglycaemia rests class cally on the demonstration of Whipple's triad of hypogly caemic symptoms after meals, proven hypoglycaemia a the time of symptoms and the relief of symptoms by th ingestion of glucose. Patients with reactive hypoglycae mia will often show delayed, exaggerated insuli secretion (termed 'dysinsulinism') in response to an ora glucose tolerance test especially if the aetiology gastrointestinal disease or 'idiopathic'.[6–8] Also, abnor malities of glucagon response to hypoglycaemia hav been described along with the dysinsulinism.[8, 9] Som patients with reactive hypoglycaemia demonstrate exag gerated gastrointestinal peptide (GIP) response to oral bu not intravenous glucose, the implication being that th increased GIP response results in the dysinsulinism observed in these patients.[10]

Our patient is unusual in several respects. Firstly he displayed marked dysinsulinism and subsequen hypoglycaemia in response to both oral and intravenou glucose loads, implying that stimulation of excessive GI release is not the underlying defect. The absent glucago response to hypoglycaemia seen in this patient is furthe evidence of a disorder of pancreatic islet cell inter regulation as the underlying defect. For example, deficiency of, or insensitivity of, the paracrine respons to native somatostatin could be postulated.

Secondly, we have demonstrated a previously unknow abnormality in the hepatic microsomal glucose-6-phos

phatase activity in this patient's liver biopsy. Hepatic glucose-6-phosphatase plays a vital part in glucose homeostasis[11] and is known to exist as a multicomponent protein system which it has only recently been possible to study on small liver samples.[3] It is not possible at present to identify the inhibitor of the glucose-6-phosphate translocase (T_1) indicated by this patient's results (Table 2). There are no known physiological inhibitors of T_1, but it is possible that the inhibitor of T_1 present in this patient is insulin, C-peptide or another peptide involved in insulin metabolism.

The pattern of reactive hypoglycaemia in this report is unlike that of fructose 1:6 diphosphatase deficiency where hypoglycaemia can be induced by fructose, alanine, and glycerol, but not by hyperglycaemia. These individuals usually show normal response to a glucagon test after 12 h fasting, but an absent response after 14 h fasting.

It is possible that the occurrence of reactive hypoglycaemia and the demonstrated hepatic glucose-6-phosphatase abnormality is a chance association in this patient. The hepatic enzyme abnormality will worsen the reactive hypoglycaemia as hepatic glucose output will not be able to increase in a normal fashion in response to normal stimuli (such as the glucagon test, Figure 3). Interestingly glucose-6-phosphatase activity has recently been demonstrated in pancreatic islet cells, where its physiological role is unclear.[12] If the same abnormality is present in the pancreatic as in the hepatic glucose-6-phosphatase enzyme system, it might imply a role for glucose-6-phosphatase in pancreatic islet cell response to blood glucose levels.

Recently somatostatin infusions have been shown to improve idiopathic reactive hypoglycaemia during an OGTT[8] and the present study suggests that octreotide may also be effective. Octreotide has been shown to be effective in the short-term control of hyperinsulinaemia due to benign and malignant insulinomas in adults and longer-term in infants with nesidioblastosis.[13–15] Our patient developed unpleasant abdominal discomfort after the single dose of 100 μg octreotide and it is planned to use octreotide only should he not respond to, or should he develop side-effects to diazoxide.

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Amiloride activation of hepatic microsomal glucose-6-phosphatase; activation of T1?

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Key words: Glucose-6-phosphatase; Microsome; Glucose 6-phosphate transport; Amiloride

e mechanism of activation of hepatic microsomal glucose-6-phosphatase (EC 3.1.3.9) in vitro by amiloride has been estigated in both intact and fully disrupted microsomes. The major effect of amiloride is a 4.5-fold reduction in the of glucose-6-phosphatase activity in intact diabetic rat liver microsomes. Amiloride also decreased the K_m of cose-6-phosphatase activity in intact liver microsomes isolated from starved rats 2.5-fold. Kinetic calculations, direct yme assays and direct transport assays all demonstrated that the site of amiloride action was T1, the hepatic rosomal glucose 6-phosphate transport protein. This is, to our knowledge, the first report of an activation of any of proteins of the multimeric hepatic microsomal glucose-6-phosphatase complex.

oduction

Hepatic glucose-6-phosphatase (EC 3.1.3.9) is a mul-mponent enzyme complex comprising the glucose-hosphatase enzyme, with its catalytic site in the ıen of the endoplasmic reticulum (ER) [1], a regu-ory calcium binding protein termed 'stabilising' pro-ı [2 and 3], and three transport proteins which allow influx of glucose 6-phosphate and the efflux of ction products, inorganic phosphate and glucose from lumen of the ER (T1, T2 and T3, respectively) [4–8]. Glucose-6-phosphatase catalyses a crucial step in the ase of glucose from the liver – a function which is l for maintaining blood glucose levels (see Refs. 9 l 10 for reviews). Kinetic analysis of the glucose-6-sphatase activity in intact and disrupted microsomes indicated that glucose 6-phosphate transport is e-limiting in hepatic glucose 6-phosphate hydrolysis Nothing is known about the regulation of T1, but its ation makes it a possible target for manipulation in treatment of diabetes mellitus, where hepatic glu-e output and glucose-6-phosphatase activity are both kedly increased. Also T1 transport capacity is more straining in diabetic than in starved liver micro-nes [4].

While investigating the effects of pharmacological agents on T1, we studied the diuretic drug, amiloride. This compound produced a profound decrease in the K_m of glucose-6-phosphatase in intact hepatic microsomes, which was shown to be due to increased transport capacity of T1.

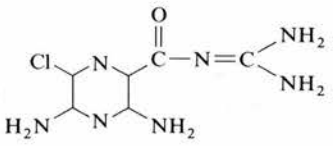
This is, to our knowledge, the first report of an activator of any of the hepatic microsomal glucose-6-phosphatase proteins and, we believe, alters the interpretation of the mechanisms involved in the regulation of this multimeric enzyme complex.

Materials and Methods

Chemicals

Glucose 6-phosphate (monosodium salt) and Cocktail T (scintillant) were obtained from BDH Chemicals, Poole, U.K. BM-Test-Glycemie strips were from the Boehringer (London, U.K.) Mannose 6-phosphate (disodium salt), amiloride hydrochloride (3,5-diamino-6-chloro-*N*-(diaminomethylene)pyrazine carboxamide),

The structure of amiloride hydrochloride



histone 2A and streptozotocin were from Sigma, Poole, U.K. Cacodylic acid, also from Sigma, was recrystallised from 95% ethanol. [U-^{14}C]Glucose 6-phosphate from Amersham International, Amersham, U.K. and

reviation; ER, endoplasmatic reticulum.

espondence: A. Burchell, Department of Obstetrics and aecology, Ninewells Hospital and Medical School, Dundee, DD1 , U.K.

nitrocellulose from Schleicher & Schuell, Dassel, F.R.G. All other chemicals were analytical grade reagents. Amiloride solution was made fresh in 10% dimethyl/sulphoxide and to volume in buffered cacodylate (pH 6.5).

P values were calculated from *t*-tests with pooled analysis of variance using Microstat (Release 4.0) software, Ecosoft, IN, U.S.A.

Microsomal preparation

Microsomes were prepared from livers of male Wistar rats in buffered sucrose/Hepes (pH 7.4) as previously described [11]. Diabetes was induced by a single tail vein injection of streptozotocin (75 mg/kg body weight) in buffered citrate (pH 4.5). Only animals in which the development of diabetes was confirmed 48 h later by the measurement of blood sugar concentration were used [12]. Starved rats were killed after a 16 h fast.

Assays

Glucose-6-phosphatase and mannose-6-phosphatase activities were assayed and calculated as in Ref. 13 and are expressed as μmol P_i released/min per mg microsomal protein. Amiloride was added to the substrate prior to the addition of microsomes. Control assays contained 1% dimethyl sulphoxide. Microsomes isolated from liver homogenates and not treated further prior to assay are referred to as untreated, and are a mixture of intact and disrupted microsomes. The proportion of intact microsomes was determined by assays of low K_m mannose-6-phosphatase activity, which is only expressed in disrupted microsomes [14]. All the microsomal preparations used in this paper were more than 89% intact in both the presence or absence of amiloride. All the undisrupted values reported in this paper are intact values, which eliminates the large errors in activity measurements which can occur if even a small proportion of microsomes are disrupted.

Glucose 6-phosphate transport (T1) activity was measured at 20 °C, using a modification of the assay described by Waddell and Burchell [15,16]. Starved rat liver microsomes were diluted 1 : 100 in cacodylate buffer (pH 6.5) and then added to the glucose 6-phosphate incubation mix (buffered to pH 6.5 with 40 mM cacodylate) containing the indicated amount of amiloride or 1% dimethylsulphoxide (as control). 0.25 ml aliquots were drawn at each time point and the reaction was stopped by rapid filtration through a nitrocellulose filter using a Bio-Rad dot blot apparatus. In each 0.25 ml aliquot there was 1 mM glucose 6-phosphate including tracer [U-^{14}C]glucose 6-phosphate (0.5 μCi/ml) and 0.02 mg microsomal protein. After drying the nitrocellulose, the individual wells were cut out as 1×1 cm squares and suspended in scintillant, and the amount of retained radioactivity was counted using a liquid scintillation counter. The background radioactivity due to the wetting of the membrane by incuba
mix and by nonspecific binding to microsomes
estimated by control incubations containing fully
rupted microsomes as blanks, as previously descri
[16]. The values obtained using fully disrupted mi
somes were subtracted from the counts obtained
intact microsomes to give the radioactivity taken
into the microsomal vesicle. The uptake via T1
calculated as described [15] and expressed in nm
glucose 6-phosphate per mg microsomal protein.
tein concentrations were estimated by the metho
Lowry as modified by Peterson [17].

Results

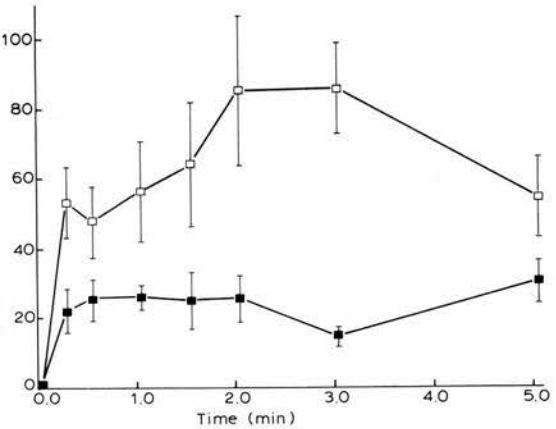
The glucose-6-phosphatase activity in intact mi
somes is a measure of the combined rates of glucos
phosphatase enzyme and the three transport protein
wide range of amiloride concentrations were adde
glucose-6-phosphatase assays, and 5 mM amiloride
found to give the maximal effect (data not shown).
addition of 5 mM amiloride to control intact fed
liver microsomes reduced the the K_m for glucos
phosphatase activity approx. 2.3-fold without appre
bly altering the V_{max}. The addition of 5 mM amilo
to intact starved rat liver microsomes reduced the
for glucose-6-phosphatase activity approx. 2.5-f
again without appreciably altering the V_{max} (see Ta
I). The addition of amiloride to intact diabetic rat l
microsomes resulted in a greater decrease in the K_m
glucose-6-phosphatase activity (approx. 4.5-fold).
K_m of intact microsomes is contributed to by the
zyme and transport proteins T1, T2 and T3. Disrup

TABLE I

Glucose-6-phosphatase activity in fed, starved and diabetic rat microsomes

Data are the mean ± S.E. from at least four separate preparat
Each preparation comprised six rat livers. Differences calculated
control values. * $P = 0.01$; ** $P < 0.01$.

	Intact microsomes		Disrupted microsom	
	K_m (mM)	V_{max} (μM/min per mg)	K_m (mM)	V_{max} (μM/m per mg)
Fed				
Control	3.3 ± 0.4	0.09 ± 0.02	0.5 ± 0.1	0.12 ± 0.
1 mM Amiloride	1.8 ± 0.1 **	0.09 ± 0.02	0.5 ± 0.2	0.11 ± 0.
5 mM Amiloride	1.4 ± 0.2 **	0.11 ± 0.02	0.5 ± 0.1	0.12 ± 0.
Starved				
Control	3.8 ± 0.5	0.18 ± 0.02	1.2 ± 0.5	0.32 ± 0.
1 mM Amiloride	2.3 ± 0.4	0.14 ± 0.01	0.9 ± 0.4	0.28 ± 0.
5 mM Amiloride	1.6 ± 0.3 **	0.17 ± 0.01	0.7 ± 0.2	0.25 ± 0.
Diabetic				
Control	5.2 ± 0.7	0.28 ± 0.05	1.6 ± 0.3	0.66 ± 0.
1 mM Amiloride	2.4 ± 0.3 **	0.22 ± 0.01	1.2 ± 0.2	0.50 ± 0.
5 mM Amiloride	1.2 ± 0.2 **	0.22 ± 0.02	0.8 ± 0.1 *	0.43 ± 0.



1. The effect of amiloride on glucose 6-phosphate uptake into osomal vesicles. Nanomoles of glucose 6-phosphate/mg micro- l protein taken up into microsomes in the presence (□) and ce (■) of 5 mM amiloride. Each point is the mean (±S.E.) of eight (amiloride) or seven (control) assays.

icrosomes removes the controlling influence of the e transport proteins and therefore is a direct mea- of the activity of the glucose-6-phosphatase en- e. Amiloride had a much smaller effect on the ity of the glucose-6-phosphatase enzyme in fully starved or diabetic disrupted microsomes, than it n the respective intact microsomes (Table I).

irect measurement of glucose 6-phosphate trans- into microsomal vesicles with and without 5 mM oride is shown in Fig. 1. The overall amount of abel (a mixture of [^{14}C]glucose 6-phosphate sub- e and [^{14}C]glucose product) inside the lumen of the osomal vesicles is increased in the presence of oride.

ussion

here have been many reports of inhibitors of the in components of rat hepatic microsomal glucose- osphatase but very few reports of activators (see 18 for a recent review). The only compounds ted to activate glucose-6-phosphatase in intact mi- omal vesicles in vitro were polyamines [19–21] and ne [22]. These compounds have recently been shown crease glucose-6-phosphatase activity by disrupting osomal vesicles and removing the rate limitations sed on glucose-6-phosphatase activity by the trans- proteins rather than by directly interacting with of the protein components of the glucose-6-phos- se system [23]. The intactness of the microsomal es in the assay reported in Table I was not signifi- y altered by the presence of amiloride, and the ntage of intact microsomes in all the untreated osomal preparations used was more than 89%. In ion, all the data shown have been corrected (as ibed in Materials and Methods) for the percent intactness. Therefore, the effects of amiloride on glucose-6-phosphatase activity in intact microsomes must be due to a direct effect on one or more of the proteins of the glucose-6-phosphatase system. Amiloride has a small effect on the K_m of the glucose-6-phosphatase enzyme in fully disrupted hepatic microsomes (Table I) and amiloride has been reported not to cross cell membranes [24]. The decrease in the K_m of glucose-6-phosphatase activity in intact microsomes could not, therefore, be caused by a direct effect of amiloride on the glucose-6-phosphatase enzyme itself. The most likely site of amiloride action therefore seems to be T1, the glucose 6-phosphate transport protein.

Glucose-6-phosphatase enzyme activity is higher in diabetic rat liver microsomes than in starved or fed rat liver microsomes (see Table I) as previously reported [4,9,12 and 14]. This is caused by the presence of high levels of the catalytic subunit of glucose-6-phosphatase in diabetic rat liver microsomes [12]. The transport capacity of T1 is thought not to alter significantly in fed, starved or diabetic rat liver microsomes [4]. Hence, as the V_{max} of the enzyme increases from fed to diabetic liver microsomes, T1 becomes more rate limiting and cannot transport enough glucose-6-phosphate into the lumen for the more active glucose-6-phosphatase enzyme. This results in a fall in the concentration of glucose 6-phosphate in the lumen of the ER and hence a higher K_m in intact vesicles. Amiloride reduces the K_m of glucose-6-phosphatase activity in intact microsomes prepared from livers of fed, starved and diabetic rats to approx. the same value, a value very close to the K_m of the glucose-6-phosphatase enzyme. This is the predicted result of increasing T1 capacity beyond the potential of the enzyme to hydrolyse glucose 6-phosphate [4].

If amiloride's only effect was to increase T1 transport capacity, the V_{max} of glucose-6-phosphatase activity in intact microsomes would have risen to a value similar to the V_{max} of the glucose-6-phosphatase enzyme in disrupted microsomes in the presence of 5 mM amiloride. However, this did not occur. The V_{max} of intact microsomes from diabetic liver microsomes was 51% of the value in disrupted microsomes (see Table I). The simplest explanation is that amiloride is inhibiting T3, the glucose transport protein. Inhibition of T3 would lead to a build-up of glucose inside the lumen of the microsomes. This would explain the increase in the levels of [^{14}C]glucose + [^{14}C]glucose 6-phosphate in the lumen of microsomes in the presence of amiloride (see Fig. 1). Glucose inhibition of glucose-6-phosphatase enzyme activity was discussed in some detail in Ref. 4, where high glucose levels were found to decrease the V_{max} of the enzyme by 52% and increase the K_m by 53% (see Ref. 4, Table I). This is extremely similar to results obtained here with diabetic microsomes in the presence of 5 mM amiloride where the intact V_{max} is 49% lower

than the enzyme's and the intact K_m is 50% higher (see Table I).

Amiloride is known to inhibit several different Na^+ transport proteins, e.g., Na^+ channels, Na^+/H^+ and Na^+/Ca^+ antiporters and Na^+/glucose cotransporters (for reviews see Refs. 24–28). However, it has also been reported [4] that the transport of glucose 6-phosphate, glucose and phosphate into the lumen of hepatic microsomes do not involve co-transport with Na^+ [4]. Amiloride is also known to affect the phosphorylation states of a number of proteins because it is a non-specific inhibitor of protein kinases [29]. To date, there is to our knowledge no evidence that glucose-6-phosphatase activity in intact microsomes is altered by phosphorylation or dephosphorylation [30]. No ATP or GTP was added to our assays or microsomal preparations, and so amiloride cannot be activating T1 via inhibition of a microsomal protein kinase. Therefore, the simplest explanation is that amiloride is interacting directly with both T1 and T3.

The pharmacological concentrations of amiloride have been estimated to be 3–20 μM [24], but the maximal effect of amiloride on glucose-6-phosphatase activity is at a concentration of 5 mM. This, coupled to the fact that amiloride does not enter hepatocytes, explains why amiloride does not affect glucose tolerance in man. However, the fact that T1 can be activated by amiloride raises the possibility of designing similar pharmacological agents which could be used to regulate blood glucose at this site.

Acknowledgements

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APPENDIX B

POST MORTEM REPORT ON CASE 1

Date of death 01 10 86 Date of Post Mortem 02 10 86

External Appearances

The body is that of a middle aged male Caucasian 63kg in weight and 172cm in height. An area of subcutaneous haematoma is present in the right hypochondrium. There is no evidence of pedal oedema and no enlarged peripheral lymph nodes are seen or palpated. There is also no evidence of muscle or fat wasting.

Internal Appearances

Cardiovascular System

The pericardial sac and cavity appear normal. The heart (380g) shows no significant abnormality of note. The left ventricle is 14cm thick and the right 2cm thick, 2cm from the apex. The valves (mitral 10cm, aortic 7cm, pulmonary 8cm and tricuspid 11cm) appear unremarkable. The coronary arteries show mild atheroma and are patent throughout their dissected courses. The aorta and major arteries to the head and neck, upper limbs and lower limbs show only mild atheroma. The Circle of Willis appears unremarkable as do the pulmonary vasculature and the systemic venous system.

Respiratory System

Both pleural cavities appear normal. The trachea and main bronchi show no abnormalities of note. The lungs (left 650g, right 670g) are markedly congested and oedematous. There is no evidence of a chest infection.

Gastrointestinal System

The tongue is healthy. The oesophagus stomach, small and large intestines show no obvious abnormalities. The gall bladder appears normal. Free bile flow can be elicited and the bile duct is probe patent. The pancreas (420g) appears unremarkable. The liver (6,000g) is massively enlarged and fills most of the abdominal cavity. A large tumour measuring 140mm in maximum dimension is present in the right lobe. The rest of the liver shows a variegated appearance with numerous yellowish nodules present within the whole of the parenchyma.

Genitourinary System

The kidneys (right 210g, left 220g) are heavy and enlarged for the size of the patient and are pale. The ureters and bladder appear unremarkable. The prostate and testes are of normal sizes.

Central Nervous System

The meninges appear normal. The brain (1,440g) shows no obvious abnormality in serial slices.

Endocrine System

The pituitary, parathyroids, thyroid (20g) and adrenals (10g together) show no obvious abnormalities.

Reticulo-Endothelial System

The spleen (440g) is enlarged and markedly congested. A single enlarged lymph node is identified. The thymus is atrophic.

Musculo-Skeletal System

No obvious abnormality is seen in this system.

Comment

Autopsy shows marked bilateral pulmonary congestion and oedema. There is a large neoplasm involving the right lobe of the liver with numerous nodules in the rest of the parenchyma. The liver is massively enlarged. The spleen is also greatly enlarged

and markedly congested. Both kidneys are also enlarged and heavy. The appearances of the liver and kidneys are in keeping with glycogen storage disease. Sections were taken for histology and the histological report followed later.

HISTOLOGY REPORT

Lungs

Sections show pulmonary oedema and numerous tumour emboli in pulmonary arteries and arterioles.

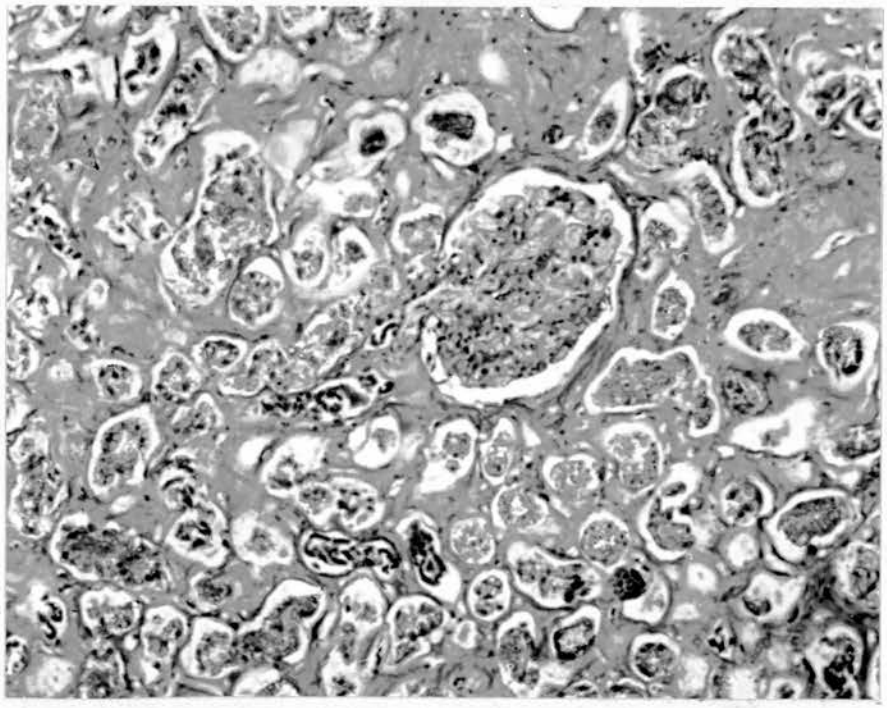
Liver

Sections show diffuse infiltration by primary liver cell carcinoma with a very variable pattern comprising areas of clear cells, pseudoacinar structures and reticular pattern. The uninvolved areas show moderate steatosis and rarefaction of the cytoplasm of "non-fat containing" hepatocytes. The nuclei are large with a central nonstaining area in places. There are large areas of necrosis. Sections of liver fixed in Rossman's fluid and stained with PAS and PAS and diastase show massive accumulation of glycogen in hepatocytes not involved in the neoplastic process, with some nuclei also containing glycogen. The neoplastic hepatocytes contain glycogen to a lesser degree. (A photograph of the liver histology is shown in Figure 3.1 & 3.2)

Kidneys

Sections show ischaemic damage with some sclerotic glomeruli. There is an increase in the interstitial fibrous connective tissue especially in the medulla. The kidneys are largely autolytic but recognisable proximal tubules show cytoplasmic vacuolation with pale empty nuclei. PAS with and without diastase of sections from kidney blocks fixed in Rossman's fluid show increased glycogen in the cytoplasm and also in the nuclei of the tubules.

a



b

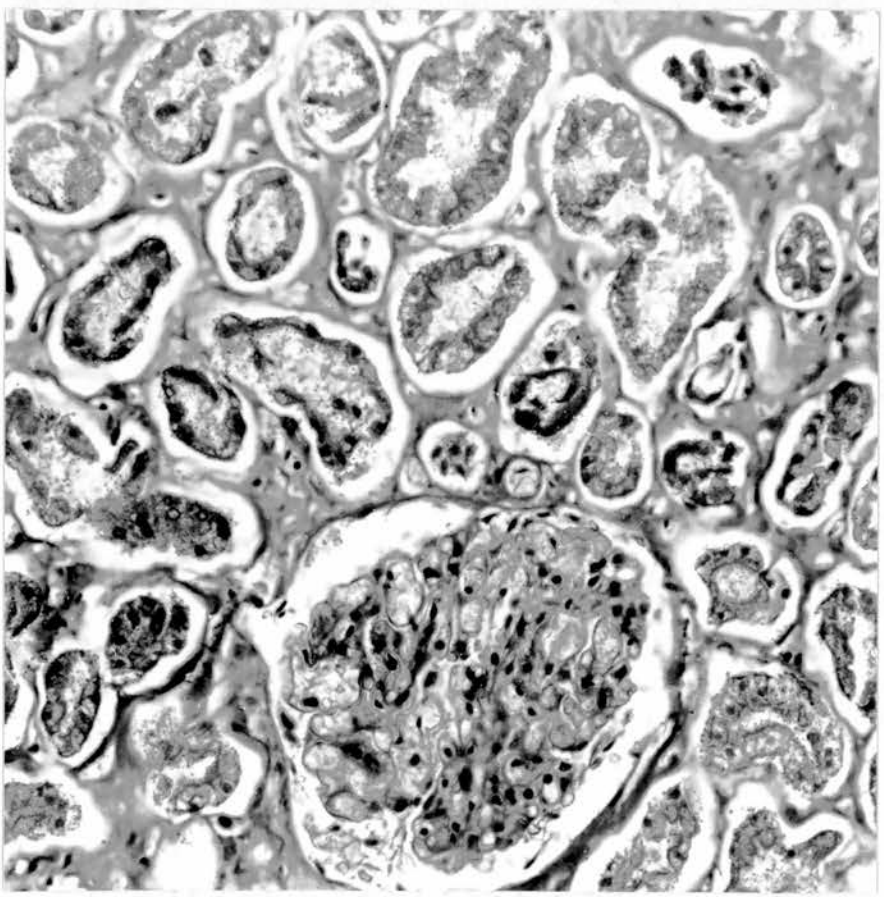


Figure B.1
Photographs of sections of kidney taken at post mortem. (a) x75 (b) x250. For details of histology see text above.

Spleen

This is markedly congested and shows an increased number of macrophages in the red pulp.

Skeletal Muscle

Sections show no remarkable features and no excess of glycogen can be seen with special stains on blocks fixed in Rossman's fluid.

Brain

No excess glycogen can be demonstrated.

Lymph node

This shows reactive changes only.

Comment

Histological examination of autopsy material shows pulmonary oedema and extensive tumour emboli in the pulmonary arterial tree. There are large amounts of glycogen in hepatocytes and proximal tubular cells of the kidneys in excess of normal as evidenced by glycogen in the nuclei. No excess of glycogen can be shown in cardiac muscle, skeletal muscle and brain tissue suggesting a type I glycogen storage disease (von Gierke's disease). There is extensive infiltration of the liver by primary liver cell carcinoma which gave rise to the tumour emboli in the lungs. Congestive splenomegaly is present.

Final Necropsy Report

1. Disease or condition leading to death:

Severe bilateral pulmonary oedema due to (or as a consequence of) **Extensive tumour emboli** due to (or as a consequence of) **Primary liver cell carcinoma.**

2. Other significant conditions contributing to the death but not related to the disease or condition causing it:

Type 1 glycogen storage disease.

APPENDIX C

MOTHER AND DAUGHTER WITH TYPE IX GSD

B.1 Introduction

These two cases are included here for completeness sake as they were diagnosed at the same time as the eight cases in Chapter 3, eventhough they do not have primarily an abnormality of glucose-6-phosphatase, although the activity of the hepatic system in both is a little depressed, possibly reflecting the high glycogen stores. The assays on liver and muscle samples from both these women were performed by Mrs. Lesley Gibb.

B.2 Methods

All methods were as described in Chapter 2 above. Skeletal muscle biopsies were taken under local anaesthetic from tibialis anterior using Tilley-Henkel biopsy forceps (designed for removal of nasal polyps). The 1cm incision was closed with steristrips after 15 minutes direct pressure to secure haemostasis. Samples were transferred dry and fresh to both pathology and biochemistry laboratories.
Total phosphorylase activity (a + b) was measured in the direction of glycogen synthesis in the presence of 5'-AMP as described by Hedrick and Fisher (1963). Phosphorylase kinase activity was measured by the method of Krebs as modified by Cohen (1973). "Physiological" phosphorylase kinase activity is measured at pH6.8, and maximal activity is measured at pH 8.8. unfortunately the sensitivity of the assay does not allow discrimination between normal and half-normal activity in extracts of human biopsy samples.

B.3 Mother

Born in Malawi 270851 of mixed race parentage (father Pakistani, mother ½ caucasian and ½ negro). Four live siblings, her mother had had nine other pregnancies which had not produced live children (the exact circumstances are unclear as they were separated at a young age). The patient had had 3 pregnancies all producing live infants.

At the age of 34 she presented to a neurologist with a 2½ year history of right-sided headache and recurrent funny turns. At night these attacks (according to her husband) consisted of a cry followed by clenching of teeth and generalised shaking. During the day she described episodes of loss of memory, blankness during, for example, conversations, dropping objects and occasional acute loss of posture. There was no clear temporal relationship to food (or lack of) and the attacks at that time had been going on for 9 months. Examination was normal. An EEG showed some spike discharges from the right parietal lobe and left posterior temporal regions, but this was not felt consistent with an active interictal record. She was given carbamazepine as a therapeutic trial with little obvious benefit. Later the attacks were thought to be anxiety-related, but she gained little help from a clinical psychologist.

By 1988 (age 37 years) her attacks were persisting but by now were more clearly preceded by a craving for sweet food and had worsened since she had gone out to work and ate less regularly. She also complained of persistent dysuria and right iliac fossa/peri-umbilical pain - urine culture was negative and an IVU was normal (a right duplex ureter was noted). On examination she was thin and introverted and had palpable hard stool in her colon, however she had no abdominal organomegaly. All routine blood testing was normal (including serum liver enzymes, bilirubin and clotting studies). During a 48 hour fast she twice felt slightly unwell: examination was unremarkable and on both occasions blood glucose was 3.5 mmol/l and serum insulin levels were 10 and < 10 mU/l. At the end of the fast and after exercise

blood glucose was 4.1, insulin 13mU/l and lactate 7.3 mmol/l. Results of oral glucose tolerance test and glucagon test are shown in Table B.1.

In view of the blunt glucose response to glucagon a liver biopsy was performed. The histology and electron microscopy of the liver (including estimated glycogen stores) were normal. The results of the initial biochemical analyses (Table B.2) suggested a form of GSD but the glucose-6-phosphatase activity did not suggest that the abnormality was a type 1 GSD. A skeletal muscle (anterior tibial) biopsy was performed to further classify the abnormality. Again the histology and electron microscopy were normal. Electromyography was performed and showed some excess of high frequency components which can be seen in but are not specific for, myopathic processes. One minute of ischaemic exercise did not produce cramping, but repetitive stimulation of the right ulnar nerve did result in reduced amplitude of the evoked potentials measured in abductor digiti minimi.

Measurement of total phosphorylase (a & b) was normal in both liver (0.5 U) and skeletal muscle (1.6 U). However phosphorylase kinase activity in both tissues was reduced to approximately 10% normal (pH 6.8: liver 0.008 U; muscle 0.005 U; pH 8.6 liver 0.015 U; muscle 0.01 U).

Thus the diagnosis was type IX GSD and she responded very well to diet containing corn starch (and to the reassurance that she was not mad!).

Table B.1

Results of a) oral glucose tolerance tests and b) fasting glucagon tests in a mother and daughter with type IXc GSD.

Times given in minutes relative to the dosing time.

Glucose values given in mmol/l

a) OGTT

Mother

Time:	0	30	60	90		
Glucose:	4.4	7.1	6.9	5.2		

Daughter

Time:	0	30	60	90	120	180
Glucose:	4.6	7.9	6.7	7.4	9.1	6.0

b) Glucagon test

Mother

Time:	0	30	60	90	120
Glucose:	4.7	6.9	4.0	2.9	3.0

Daughter

Time:	0	30	60	90	120
Glucose:	4.5	6.1	4.7	2.9	2.8

Table B.2

Results of glucose-6-phosphatase assays and biochemical glycogen estimations on liver biopsy samples from a mother and daughter with type IXc GSD.

	Intact microsomes		Disrupted microsomes		Glycogen content
	Vmax (nmoles/min/mg)	Km (mM)	Vmax (nmoles/min/mg)	Km (mM)	(mg glycogen /g wet weight liver)
Mother	0.16	2.1	0.3	0.4	70
Daughter	0.1	2.7	0.14	0.4	62
Mean control values (n=60)	0.23±0.02	2.6±0.03	0.35±0.03	0.8±0.08	<40

B.4 Daughter

Born 110771. Normal birth after normal pregnancy; birth weight unknown. Separated from her mother at a relatively early age as her mother and father divorced. She had no knowledge of her mother's symptoms when she presented at age 17 with episodic dizziness and light-headedness. These symptoms were worse just before lunch when she was at work, especially if she had missed breakfast. The symptoms improved within 1 hour of taking a meal and their onset was not related to alcohol intake. Clinical examination and routine blood tests were all normal. During a 48 hour fast she had one "funny turn" when her blood glucose was estimated at 2.8 mmol/l (unfortunately on a glucose oxidase strip only). At the end of the fast and after exercise her blood glucose was 3.5 mmol/l. The blood glucose response to glucagon when fasting was blunt and an oral glucose tolerance test was unusual in showing two peaks of glucose at 30 and 120 minutes (Table B.1).
Liver biopsy was normal to light and electron microscopy, and the glucose-6-phosphatase and glycogen assays are shown in Table B.2. The glycogen stores are elevated in the liver and the glucose-6-phosphatase activity is at the low end of "normal". Muscle biopsy showed high-normal glycogen stores (19.9 mg/g muscle) on assay but was histologically normal. Total phosphorylase (a + b) activity was normal in liver (1.1 U) and muscle (5.3 U). Phosphorylase kinase activity at pH 6.8 (0.09 U liver and 0.12 U muscle) and at pH 8.6 (0.11 U liver and 0.23 U muscle) were both within the range of normal, however the assay at this level cannot distinguish normal from half-normal. In view of the glycogen accumulation and her mother's diagnosis this data was interpreted as showing this girl to have partial type IX GSD.
Her symptoms have disappeared with regular meals containing uncooked corn starch. She has one son (delivered normally after a normal pregnancy) who is hyperactive but otherwise well. She has two brothers by the same parents, both of whom are asymptomatic, but one of whom had a blunt glucose response (+2.4

mmol/l) to glucagon. The other brother's test was normal. Neither brother has had any further investigations performed and the father was untraceable.

B.5 Discussion

Type IX glycogen storage disease is well recognised as being mild and survival to adulthood is the norm (Abarbanel *et al* 1986). These two cases have several unusual features. The vagueness of the mother's symptoms may be related to her inability to describe them clearly (or of the doctors to elicit them!) but one wonders how many other people have been described as having anxiety-related symptoms and been referred to clinical psychologists. Both women developed biochemical hypoglycaemia after their glucagon test which was only seen in one of the other eight cases (Case 8) described in Chapter 3. This is not specific for any abnormality, but in time it may become clear that some types of GSD patients do develop hypoglycaemia after glucagon administration. The double peak in blood glucose levels during a standard 75g oral glucose tolerance test in the daughter is likewise noted but the aetiology and significance remain obscure. Unfortunately serum insulin levels were not measured.

Four human variants of type IX GSD are currently recognised: type IXa (autosomal recessive - abnormal liver but normal muscle PK activity); type IXb (sex-linked - abnormal liver but normal muscle PK activity [Huijing & Fernandes 1969]); type IXc (autosomal recessive - abnormal activity in liver and muscle [Lederer *et al* 1980]) and type IXd muscle PK deficiency with normal liver activity (Abarbanel *et al* 1986).

Type IXc GSD (phosphorylase kinase deficiency affecting muscle and liver) is thought to be inherited in an autosomal recessive fashion (Bashan *et al* 1981 and Lerner *et al* 1982). These two cases show that that is incorrect (at least for these two cases).

While the phosphorylase kinase activity measured in the liver sample from the daughter is low-normal rather than being clearly low, she undoubtedly has a disorder of glycogen metabolism and in view of her mother's diagnosis the chances of it being caused by a different (and unmeasured by us) liver enzyme defect are very small. The development of the daughter's siblings and child are being watched with interest.

Although the disease is usually mild, the diagnosis is an important one to recognise as understanding the illness and knowledge of dietary manipulation can save the patient a lot of distress.

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